APPENDIX A

cent of patients with AIDS were found to react with it (*38*). In contrast, HTLV-III is related to HTLV-I and -II (*31, 39*) and, by all criteria, this new virus belongs to the HTLV family of retroviruses. In addition, more than 85 percent of serum samples from AIDS patients are reactive with proteins of HTLV-III (*33*). These findings suggest that HTLV-III and LAV may be different. However, it is possible that this is due to insufficient characterization of LAV because the virus has not yet been transmitted to a permanently growing cell line for true isolation and therefore has been difficult to obtain in quantity.

The transient expression of cytopathic variants of HTLV in cells from AIDS patients and the previous lack of a cell system that could maintain growth and still be susceptible to and permissive for the virus represented a major obstacle in detection, isolation, and elucidation of the precise causative agent of AIDS. The establishment of T-cell populations that continuously grow and produce virus after infection opens the way to the routine detection of cytopathic variants of HTLV in AIDS patients and provides the first opportunity for detailed immunological (*31, 33*) and molecular analyses of these viruses.

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Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and at Risk for AIDS

Abstract. *Peripheral blood lymphocytes from patients with the acquired immunodeficiency syndrome (AIDS) or with signs or symptoms that frequently precede AIDS (pre-AIDS) were grown in vitro with added T-cell growth factor and assayed for the expression and release of human T-lymphotropic retroviruses (HTLV). Retroviruses belonging to the HTLV family and collectively designated HTLV-III were isolated from a total of 48 subjects including 18 of 21 patients with pre-AIDS, three of four clinically normal mothers of juveniles with AIDS, 26 of 72 adult and juvenile patients with AIDS, and from one of 22 normal male homosexual subjects. No HTLV-III was detected in or isolated from 115 normal heterosexual subjects. The number of HTLV-III isolates reported here underestimates the true prevalence of the virus since many specimens were received in unsatisfactory condition. Other data show that serum samples from a high proportion of AIDS patients contain antibodies to HTLV-III. That these new isolates are members of the HTLV family but differ from the previous isolates known as HTLV-I and HTLV-II is indicated by their morphological, biological, and immunological characteristics. These results and those reported elsewhere in this issue suggest that HTLV-III may be the primary cause of AIDS.*

The acquired immunodeficiency syndrome known as AIDS was initially recognized as a separate disease entity in 1981 (*1*). Groups reported to be at risk for AIDS include homosexual or bisexual males (about 70 percent of reported cases), intravenous drug users (about 17 percent of cases), and Haitian immigrants to the United States (about 5 percent of cases). Also at risk are heterosexual contacts of members of the highest risk group, hemophiliacs treated with blood products pooled from donors, recipients of multiple blood transfusions, and infants born of parents belonging to the high-risk groups (*2*). AIDS is diagnosed as a severe, unexplained, immune deficiency that usually involves a reduction in the number of helper T lymphocytes and is accompanied by multiple opportunistic infections or malignancies. A number of other clinical manifestations, when occurring in members of a group at risk for AIDS, are identified as its prodrome (pre-AIDS). These include unexplained chronic lymphadenopathy or leukopenia involving a reduction in the number of helper T lymphocytes (*1, 2*). The increasing incidence of this disease, the types of patients affected, and other epidemiological data suggest the existence of an infectious etiologic agent that

can be transmitted by intimate contact or by whole blood or separated blood components (*2*). As indicated by Popovic *et al.* (*3*), we and others have suggested that specific human T-lymphotropic retroviruses (HTLV) cause AIDS (*4*, *5*). Many properties of HTLV are consistent with this idea (*6*).

An association of members of the HTLV family with T lymphocytes from some AIDS or pre-AIDS patients was reported previously. For example, the first subgroup of HTLV to be characterized, HTLV-I, was isolated recently from T cells from about 10 percent of AIDS patients, and a virus related to HTLV-II was isolated from one AIDS patient (*4*). Another HTLV isolate was obtained from the lymph nodes of a patient with lymphadenopathy and at risk for AIDS (*7*). This isolate has been difficult to grow in quantities sufficient to permit its characterization. HTLV proviral DNA was detected in T lymphocytes from two additional AIDS patients (*8*) and HTLV-related antigens were found in another two patients (*4*). Studies in which disrupted HTLV-I or the purified structural proteins (p24 or p19) were used to detect antibodies in serum samples from patients with AIDS and pre-AIDS indicated that 10 to 15 percent of the patients had been exposed to HTLV-I (*9*). Essex and his co-workers, using HTLV-infected T-lymphocyte cultures to detect antibody in serum samples, found that about 35 percent of patients with AIDS and pre-AIDS had been exposed to HTLV (*5*). Further studies suggested that at least some of the antigens detected in this system were products of the genome of a member of the HTLV family (*10*), but it was not known whether the antibodies were specifically against HTLV-I, HTLV-II, or a virus of a different subgroup.

With the availability of large quantities of HTLV-III (*3*), it became possible to develop specific immunological reagents that would facilitate its characterization. HTLV-III was found to share many properties with other HTLV isolates (*6*), but it was morphologically, biologically, and antigenically distinguishable (*3*, *11*). Here we describe the detection and isolation of HTLV-III from a large number of patients with AIDS and pre-AIDS.

For these studies we used cell culture conditions previously developed in our laboratory for the establishment of T lymphocytes in culture and for the detection and isolation of HTLV-I and HTLV-II from leukemic donors (*12*). Evidence for the presence of HTLV-III included: (i) viral reverse transcriptase (RT) activity (*12*) in supernatant fluids; (ii) transmission of virus by coculturing T cells with irradiated donor cells or with cell-free fluids (*3*, *13*); (iii) observation of virus by electron microscopy (*12*, *13*); and (iv) the expression of viral antigens in indirect immune fluorescence assays using serum from a patient positive for antibodies to HTLV-III as described (*5*, *11*), or antisera prepared against purified, whole disrupted HTLV-III (*11*). Cells and supernatant fluids were also monitored for the expression of HTLV-I and HTLV-II by using antibodies to the viral structural proteins p19 and p24 and by indirect immune fluorescence and radioimmunoprecipitation procedures (*14*).

As summarized in Table 1, we found

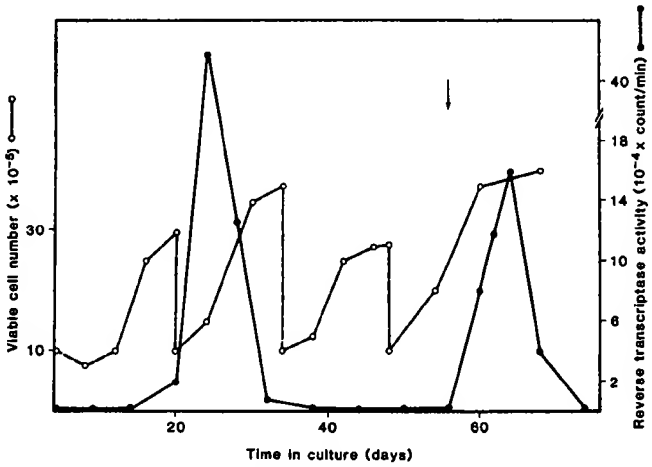

Fig. 1. Reverse transcriptase activity from lymphocytes established in cell culture from a patient with pre-AIDS. Viable cell number and Mg^{2+}-dependent RT activity were determined by established procedures (*13*). Symbols: ○, viable cell number in 1.5 ml of growth medium; ●, RT in 5 μl of fivefold concentrated conditioned medium sampled at the indicated time. A sudden vertical drop in the dashed curve indicates the time of subculturing of cells to the indicated cell number. Arrow indicates the time of addition of rabbit antiserum to α-interferon to a portion of the cultured cells (also see legend to Table 1).

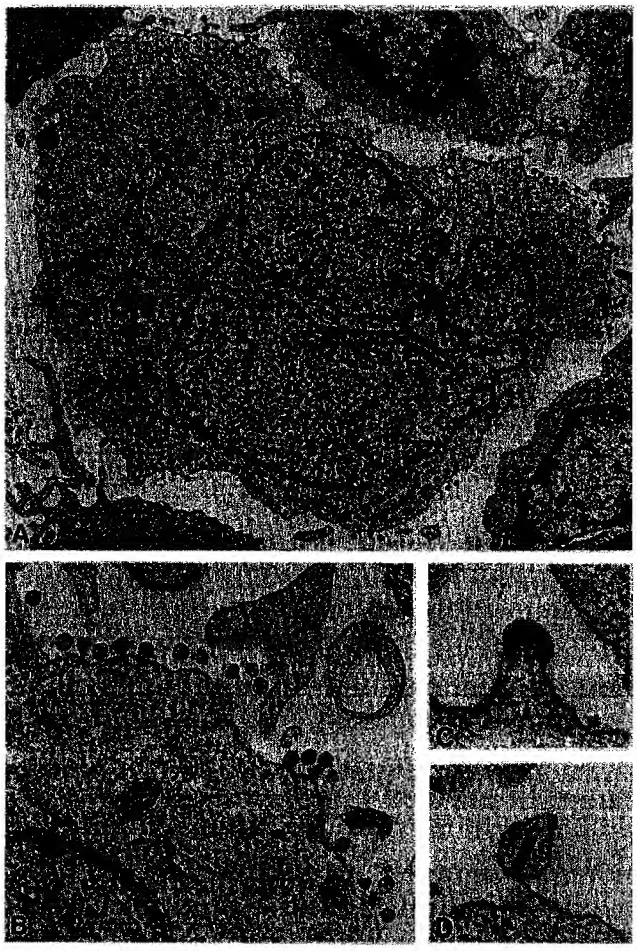

Fig. 2. Transmission electron micrographs of fixed, sectioned lymphocytes from a patient with pre-AIDS. (A) ×10,000; (B) ×30,000; (C and D) ×100,000.

HTLV-III in 18 of 21 samples from patients with pre-AIDS, from three of four clinically normal mothers of juvenile AIDS patients, three of eight juvenile AIDS patients, 13 of 43 of adult AIDS patients with Kaposi's sarcoma, and 10 of 21 adult AIDS patients with opportunistic infections. Virus was detected in only one of 22 samples from clinically normal, nonpromiscuous homosexual males believed to be at only moderate risk for AIDS. It is interesting, however, that 6 months after these tests were conducted the one positive normal homosexual subject developed AIDS. In no instance, 0 of 115, was virus detected in or isolated from cells of the normal volunteers. Samples from 15 of these were tested under rigorously controlled conditions, which included addition of antibody to α-interferon.

Primary cells from patients usually produce virus for 2 to 3 weeks (Fig. 1). After this time the production of virus declines even though the culture may contain actively replicating cells that can be maintained for long periods in the presence of added T-cell growth factor (TCGF). In some instances virus release can be reinitiated by the addition of antibody to α-interferon (Fig. 1). The HTLV-III–producing cell cultures were characterized by established immunological procedures (*13*). They were predominantly T lymphocytes (E rosette receptor–positive, $OKT3^+$ and $Leu1^+$) with a helper-inducer phenotype ($OKT4^+$ and $Leu3^+$).

The fairly uniform morphological appearance of HTLV-III is shown in Fig. 2. The diameter of the virus is 100 to 120 nm, and it is produced in high numbers from infected cells by budding from the cell membrane. A possibly unique feature of this virus is the cylindrical shaped core observed in many mature virions.

The incidence of virus isolation reported here probably underestimates its true incidence since many tissue specimens were not received or handled under what we now recognize as optimal conditions (*15*). This is particularly so for the samples received from late-stage AIDS patients. Such samples usually contain many dying cells and very few viable T4 lymphocytes. However, a high proportion of patients with AIDS and pre-AIDS have circulating antibody to HTLV-III (*11*).

The HTLV-III produced by cultured T cells from patients with AIDS and pre-AIDS is highly infectious and can be readily transmitted to fresh umbilical cord blood and adult peripheral blood or bone marrow lymphocytes. The production of HTLV-III by these cells is transient, often declining to undetectable levels by 2 to 3 weeks after infection (data not shown). The transmission of HTLV-III to an established T-cell line (*3*), however, now makes possible its production in large quantities for detailed analyses and for development of reagents for its detection (*3*, *11*).

That the viruses we have named HTLV-III belong to the HTLV family is indicated by their T cell tropism, Mg^{2+}-dependent RT of high molecular weight, antigenic cross-reactivity with HTLV-I and -II (*11*), cytopathic effects on T lymphocytes (*3*), and their morphological appearance in the electron micrograph. HTLV-III also contains some structural proteins similar in size to those of other members of the HTLV family (*11*).

These studies of HTLV-III isolates from patients with AIDS and pre-AIDS and from some healthy individuals at risk for AIDS provide strong evidence of a causative involvement of the virus in AIDS.

Table 1. Detection and isolation of HTLV-III from patients with AIDS and pre-AIDS. Peripheral blood leukocytes were banded in Ficoll-Hypaque, incubated in growth medium (RPMI 1640, 20 percent fetal bovine serum, and 0.29 mg of glutamine per milliliter) containing phytohemagglutinin (PHA-P; 5 μg/ml) for 48 hours at 37°C in a 5 percent CO_2 atmosphere. They were then refed with growth medium containing 10 percent purified T-cell growth factor (TCGF). Cells and conditioned media from these lymphocytes were assayed for the presence of HTLV-III. Samples exhibiting more than one of the following were considered positive: repeated detection of a Mg^{2+}-dependent reverse transcriptase activity in supernatant fluids; virus observed by electron microscopy; intracellular expression of virus-related antigens detected with antibodies from seropositive donors or with rabbit antiserum to HTLV-III; or transmission of particles, detected by RT assays or by electron microscopic observation, to fresh human cord blood, bone marrow, or peripheral blood T lymphocytes. All isolates are distinguishable from HTLV-I or HTLV-II by several criteria and are classified as HTLV-III on the basis of similar morphological features observed by electron microscopy (Fig. 1); similar cytopathic effects (*3*); antigenic cross-reactivity (*11*); and nucleic acid analysis (*16*).

Diagnosis*	Number positive for HTLV-III	Number tested	Percent positive
Pre-AIDS	18	21	85.7
Clinically normal mothers of juvenile AIDS patients	3	4	75.0
Juvenile AIDS	3	8	37.5
Adult AIDS with Kaposi's sarcoma	13	43	30.2
Adult AIDS with opportunistic infections	10	21	47.6
Clinically normal homosexual donors	1	22	4.5
Clinically normal heterosexual donors	0	115	0

*With the exception of the normal heterosexual donors and some of the clinically normal mothers of juvenile AIDS patients, all others belong to one of the groups of people identified as being at risk for AIDS (homosexual males, intravenous drug users, Haitian immigrants, heterosexual contacts of members of a group at risk, hemophiliacs treated with pooled blood products, recipients of multiple blood transfusions, and infants born of parents belonging to other groups at risk). Pre-AIDS includes patients with unexplained chronic lymphadenopathy and leukopenia, with an inverted T4 (helper)/T8 (suppressor) lymphocyte ratio. The clinically normal, nonpromiscuous, homosexual subjects are from Washington, D.C., and are believed to be at moderate risk. The clinically normal heterosexual donors include both male and female subjects believed not to be at risk for AIDS.

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15. For virus isolation, samples of freshly drawn, heparinized peripheral blood or bone marrow, yielding a minimum of 10^7 viable cells (greater than 90 percent), are needed. These samples must contain the cells of interest, namely, $OKT4^+$ T cells, which are frequently depleted in AIDS patients.
16. S. Arya *et al.*, in preparation.

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Serological Analysis of a Subgroup of Human T-Lymphotropic Retroviruses (HTLV-III) Associated with AIDS

Abstract. *The two main subgroups of the family of human T-lymphotropic retroviruses (HTLV) that have previously been characterized are known as HTLV-I and HTLV-II. Both are associated with certain human leukemias and lymphomas. Cell surface antigens (p61 and p65) encoded by HTLV-I are frequently recognized, at low titers, by antibodies in the serum of patients with acquired immunodeficiency syndrome (AIDS) or with signs or symptoms that precede AIDS (pre-AIDS). This suggests an involvement of HTLV in these disorders. Another subgroup of HTLV, designated HTLV-III, has now been isolated from many patients with AIDS and pre-AIDS. In the studies described in this report, virus-associated antigens in T-cell clones permanently producing HTLV-III were subjected to biochemical and immunological analyses. Antigens of HTLV-III, specifically detected by antibodies in serum from AIDS or pre-AIDS patients and revealed by the Western blot technique, are similar in size to those found in other subgroups of HTLV. They include at least three serologically unrelated antigenic groups, one of which is associated with group-specific antigens (p55 and p24) and another with envelope-related (p65) proteins, while the antigens in the third group are of unknown affiliation. The data show that HTLV-III is clearly distinguishable from HTLV-I and HTLV-II but is also significantly related to both viruses. HTLV-III is thus a true member of the HTLV family.*

Members of the family of human lymphotropic retroviruses (HTLV) have the following features in common: a pronounced tropism for $OKT4^+$ lymphocytes (*1*), a reverse transcriptase (RT) with a high molecular weight (100,000) and a preference for Mg^{2+} as the divalent cation for optimal enzymatic activity (*2*, *3*), and the capacity to inhibit T cell function (*4*) or, in some cases, kill T cells (*5*). Many HTLV also have the capacity to transform infected T cells (*1*). The two major subgroups that have been characterized (*6*) are HTLV-I, which is causatively linked to certain adult T-cell malignancies (*7*), and HTLV-II, which was first identified in a patient with hairy cell leukemia (*8*).

Viruses of the HTLV family have been detected in some patients with the acquired immunodeficiency syndrome (AIDS) (*9*) or with pre-AIDS, a condition frequently progressing to AIDS (*10*). A high proportion of patients with AIDS or pre-AIDS, as well as a significant number of hemophiliacs, have antibodies in their serum that recognize a cell surface glycoprotein (gp61) that is present on certain human T cells infected with HTLV-I (*11*). Gp61 and p65, a slightly larger protein that is a homolog of gp61 and occurs in another cell line producing HTLV-I, were subsequently shown to be related to the HTLV viral glycoprotein (*12*, *13*). Studies of blood transfusion recipients who later developed AIDS and of their blood donors have revealed the presence, in the blood of the donors, of antibodies to a retrovirus of the HTLV family (*14*). These findings suggest an involvement of viruses of the HTLV family in the cause of AIDS and pre-AIDS. An involvement of HTLV-I alone appeared doubtful, however, because antibody titers to gp61 of HTLV-I in these patients are generally very low and antibodies to the structural proteins of HTLV, notably p24 and p19 (*15*), are not detectable in most AIDS patients (*16*). Instead, it seemed likely that another member of the HTLV family might be involved in the etiology of AIDS. Here we describe our studies of a group of cytopathic viruses (collectively designated HTLV-III) isolated from patients with AIDS or pre-AIDS. Isolation of these viruses was achieved by means of a novel system permitting the continuous growth of T-cell clones infected with the cytopathic types of HTLV found in these disorders (*17*). We show that antigens associated with human cells infected by HTLV-III are specifically recognized by antibodies in serum from AIDS and pre-AIDS patients, and present a preliminary biochemical and immunological analysis of these antigens.

Lysates of two immortalized and infected human T-cell clones, H4/HTLV-III and H17/HTLV-III (*17*), were tested with samples of human serum in a strip radioimmunoassay (RIA) based on the Western blot technique (*18*). The sera were from patients with AIDS or pre-AIDS, from contacts of such patients, and from homo- or heterosexual male controls. Sera from the same patients were also tested by the enzyme-linked immunosorbent assay (ELISA) with purified HTLV-III as part of a larger, systematic serologic study of the prevalence of antibodies to HTLV-III in AIDS and pre-AIDS patients (*19*).

Representative results are shown in Fig. 1. Sera from patients with AIDS or pre-AIDS, and from some homosexuals and heroin-addicts, recognized a number of specific antigens not detected by sera from heterosexual subjects. The most prominent reactions were with antigens of the following molecular weights: 65,000, 60,000, 55,000, 41,000, and 24,000. Antigens with molecular weights of approximately 88,000, 80,000, 39,000, 32,000, 28,000, and 21,000 gave less prominent reactions. The reaction with the antigen of 55,000 (p55) only occurred in sera that also recognized p24, suggesting a relationship between the two antigens.

The specificity of these reactions was

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Delineation of a Region of the Human Immunodeficiency Virus Type 1 gp120 Glycoprotein Critical for Interaction with the CD4 Receptor

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Summary

The primary event in the infection of cells by HIV is the interaction between the viral envelope glycoprotein, gp120, and its cellular receptor, CD4. A recombinant form of gp120 was found to bind to a recombinant CD4 antigen with high affinity. Two gp120-specific murine monoclonal antibodies were able to block the interaction between gp120 and CD4. The gp120 epitope of one of these antibodies was isolated by immunoaffinity chromatography of acid-cleaved gp120 and shown to be contained within amino acids 397–439. Using in vitro mutagenesis, we have found that deletion of 12 amino acids from this region of gp120 leads to a complete loss of binding. In addition, a single amino acid substitution in this region results in significantly decreased binding, suggesting that sequences within this region are directly involved in the binding of gp120 to the CD4 receptor.

Introduction

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome, AIDS (Curran et al., 1985; Weiss, 1986). The virus predominantly attacks cells of the immune system that bear the CD4 surface antigen (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et al., 1985). These cells include the helper subset of T lymphocytes as well as other CD4 cells such as macrophages (Reinherz and Schlossman, 1980). Initial evidence suggesting that CD4 might be a component of the receptor for HIV involved the use of various murine monoclonal antibodies specific for CD4 that were able to effectively block viral infection of CD4-positive cell lines (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et al., 1985, 1986b). Direct evidence has come from studies demonstrating that a specific complex is formed between the major virus envelope glycoprotein, gp120, and CD4 antigen expressed on the host cell surface (McDougal et al., 1986a). The most compelling evidence for the role of CD4 as the HIV receptor comes from recent work in which cell lines nonpermissive for HIV infection were converted into infectible cell lines following transfection with and expression of a human CD4 cDNA sequence (Maddon et al., 1986).

While these studies strongly suggest that CD4 is the HIV receptor, little is known about the interaction between CD4 and gp120. The amino acid sequence of gp120 is highly variable among HIV-1 strains, possibly reflecting the immunoselection of neutralization-resistant viruses during the course of an infection (Starcich et al., 1986; Alizon et al., 1986; Willey et al., 1986; Hahn et al., 1986). Interestingly, the hypervariable regions of gp120 are interspersed with highly conserved regions in a manner reminiscent of antibody variable domains. Since all HIV strains appear to utilize CD4 as a cellular receptor, it would thus appear that some or all of these conserved regions contribute to the interaction between gp120 and CD4.

In this paper, we demonstrate that a soluble form of the HIV-1 envelope glycoprotein binds to recombinant cell-surface CD4 with high affinity. Using this assay, we have identified gp120-specific murine monoclonal antibodies capable of inhibiting the interaction between gp120 and CD4. The epitopes recognized by these monoclonal antibodies have been mapped to a relatively conserved region near the carboxyl terminus of gp120. In vitro mutagenesis of this region of gp120 results in mutant glycoproteins that exhibit diminished binding to CD4. These experiments represent the initial identification of a region of gp120 that may directly participate in the interaction with the CD4 surface antigen.

Results

Determination of the gp120-CD4 Binding Constant

To facilitate the characterization of the interaction between gp120 and CD4, we have developed stably transfected mammalian cell lines producing high levels of each protein. We previously demonstrated the expression of a soluble, secreted form of gp120 in Chinese hamster ovary (CHO) cells (Lasky et al., 1986). This antigen was purified to homogeneity by immunoaffinity chromatography and shown to induce neutralizing antibodies when injected into rabbits and guinea pigs. In addition, a CHO cell line that constitutively expresses membrane-bound human CD4 was constructed using a similar expression system (D. Smith and D. Capon, unpublished data). Immunofluorescence analysis employing human anti-gp120 antibodies revealed that soluble gp120 was able to bind CHO/CD4 CHO cells but not untransfected CHO cells (Figure 1). Following initial binding, the antibody:gp120:CD4 complex was observed to aggregate in the plane of the plasma membrane and, ultimately, to endocytose into the cell (Figure 1). These results demonstrate that the soluble gp120 molecule is capable of binding to recombinant CD4 expressed on the surface of transfected CHO cells.

To provide a radioligand for receptor-binding studies, recombinant gp120 was iodinated in vitro using lactoperoxidase. The iodinated gp120 was largely intact after radiolabeling with small amounts of 70K and 50K degradation products evident (Figure 2). The interaction of I^{125}-gp120 with CD4 was initially analyzed by coimmunoprecip-

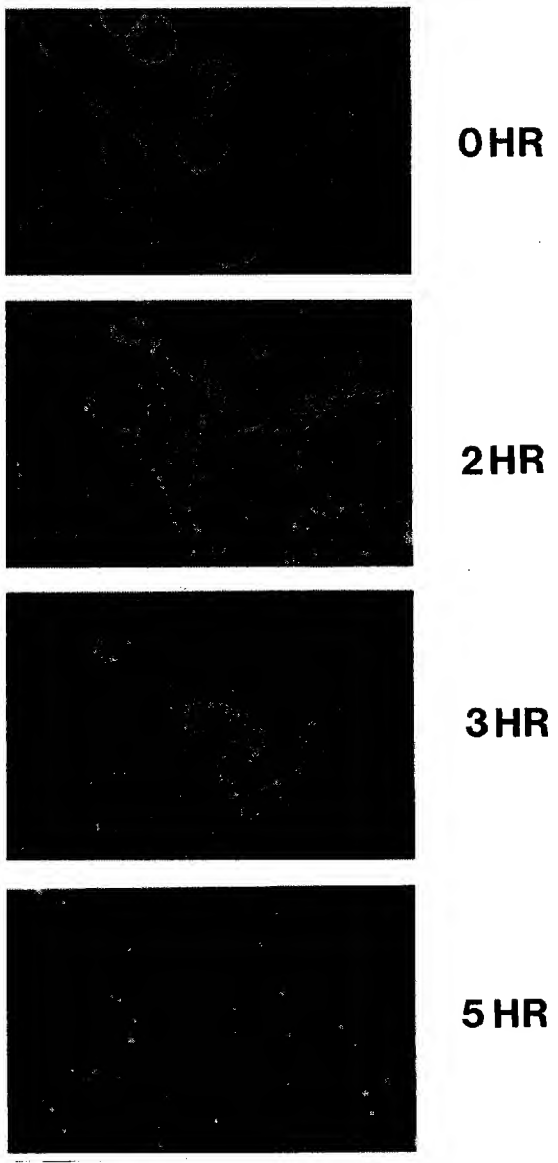


Figure 1. Binding of Recombinant gp120 to the CD4 Antigen Expressed on the Surface of CHO Cells

CHO cells transfected with a plasmid for the expression of surface CD4 were plated onto coverslips and incubated with highly purified recombinant gp120 at 4°C. The bound complex was then incubated with antibodies from an HIV-seropositive individual known to have high gp120 antibody titers. The cells were then brought to 37°C for various times, incubated with a rhodamine-labeled anti-human IgG antibody, and visualized in the fluorescence microscope. Control CHO cells not expressing surface CD4 were found to bind barely perceptible quantities of gp120 in this assay.

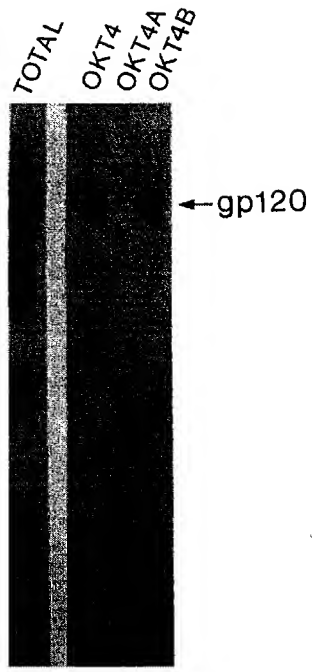


Figure 2. Coprecipitation of I^{125}-Labeled gp120 and CD4 with Various OKT4 Monoclonal Antibodies

Purified gp120 was labeled with I^{125} using the lactoperoxidase reaction to a specific activity of 0.53 nCi/ng. The isolated labeled protein was then incubated with CHO cells expressing CD4, after which the complex was solubilized in NP40, immunoprecipitated with a variety of OKT4 monoclonal antibodies, and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The first lane shows the purified I^{125}-labeled gp120 and the other lanes show the coprecipitation of the gp120–CD4 complex with the OKT4, OKT4a, and OKT4b CD4 monoclonal antibodies (Ortho Pharmaceuticals), respectively.

itation of the complex using murine monoclonal antibodies specific for CD4 (McDougal et al., 1986a). As shown in Figure 2, I^{125}-gp120 could be coimmunoprecipitated with anti-CD4 monoclonal antibodies OKT4 and OKT4B, but not with the OKT4A monoclonal antibody. This is in agreement with previous results demonstrating that the OKT4A monoclonal antibody can block virus infection but cannot coprecipitate the viral gp120–CD4 complex, whereas the OKT4 and OKT4B monoclonal antibodies do not inhibit virus infection but are able to precipitate the complex (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et al., 1985, 1986a, 1986b; Maddon et al., 1986). The interaction between soluble gp120 and the recombinant CD4 receptor is thus representative of that observed for the naturally occurring viral envelope and the receptor found on human CD4-positive cells.

The affinity constant for the gp120–CD4 interaction was examined by whole-cell saturation binding and competition binding analysis. Figure 3 shows the displacement of I^{125}-labeled gp120 binding to CHO/CD4 cells using noniodinated competitor gp120. Scatchard analysis of these data reveals a single class of binding sites with a dissociation constant (kd) of approximately 4×10^{-9} M. The binding constant measured here is comparable to that measured in other virus–receptor interactions (see Discussion). In addition, the binding is disrupted after treating gp120 at 100°C for 10 min, suggesting that higher-order structure is important in gp120–CD4 interaction (McDougal et al., 1986b; T. Gregory, unpublished data). Thus the interaction between these two recombinant proteins appears to be representative of the binding of HIV

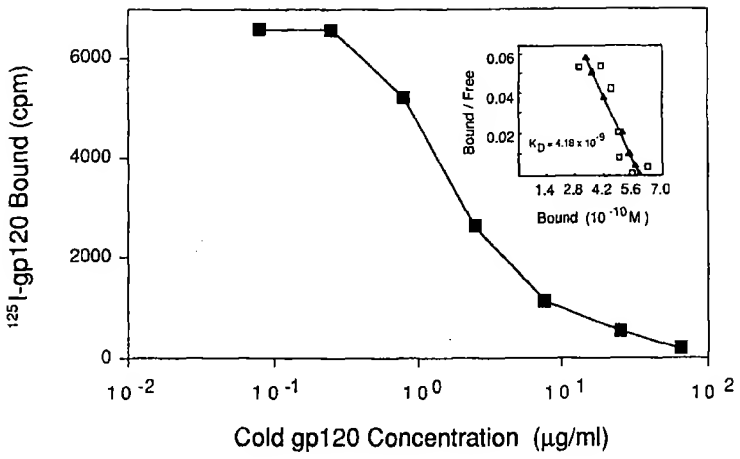


Figure 3. Competition Binding and Scatchard Analysis of the gp120–CD4 Interaction

A constant amount of I^{125}-labeled gp120 (100,000 cpm) was incubated with CHO cells expressing CD4 and an increasing amount of cold gp120 for 20 hr at 4°C. Unbound counts were washed off and the remaining bound I^{125} gp120 was measured in a gamma counter. The resultant data were fitted using the Scatplot program (D. Vandlen, Genentech, Inc.) and are shown in the inset. Nonspecific binding to CHO cells not expressing the CD4 antigen was 5% of the specific binding.

to its receptor on the surface of the cell. These results also demonstrate that a soluble form of the envelope glycoprotein, i.e., in the absence of interactions with the gp41 transmembrane protein (Veronese et al., 1985) or virus surface, is still able to bind with high affinity to CD4 (Lundin et al., 1987).

Isolation of gp120 Monoclonal Antibodies That Block the gp120–CD4 Interaction

Several monoclonal antibodies specific for gp120 were produced from mice immunized with the recombinant antigen. The resulting monoclonal antibodies were tested in the iodinated gp120–CD4 binding assay described above for their ability to block the interaction between these proteins. Of the 20 monoclonal antibodies tested, two, termed 5C2E5 and 7F11, were found to inhibit the interaction between the proteins. Figure 4 shows an inhibition experiment with one blocking gp120-specific monoclonal antibody (5C2E5) and a nonblocking gp120-specific monoclonal antibody (9F6). The figure shows that the binding of the proteins is inhibited at a relatively high 5C2E5 antibody level and over a wide range of antibody concentrations, a result that may reflect the high binding constant of gp120 for CD4. An Fab fragment of the 5C2E5 monoclonal antibody was also effective in blocking the gp120–CD4 interaction, ruling out the possibility that inhibition is due to gp120 aggregation (Figure 4). The 9F6 monoclonal antibody shown here is representative of the majority of gp120-specific monoclonal antibodies that are completely ineffective in blocking the binding reaction, even at high antibody concentrations. This figure also reconfirms that the OKT4 CD4 monoclonal antibody is ineffective in blocking the gp120–CD4 interaction whereas the OKT4a CD4 monoclonal antibody blocks this interaction effectively at low antibody concentrations, a result that agrees with previous experiments using either native gp120 binding or virus infectivity as the assay (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et al., 1985, 1986a, 1986b).

While the blocking of gp120–CD4 binding may have been due to an antibody-induced conformational change in the overall structure of gp120, the simplest interpretation of the results is that the epitope recognized by this anti-

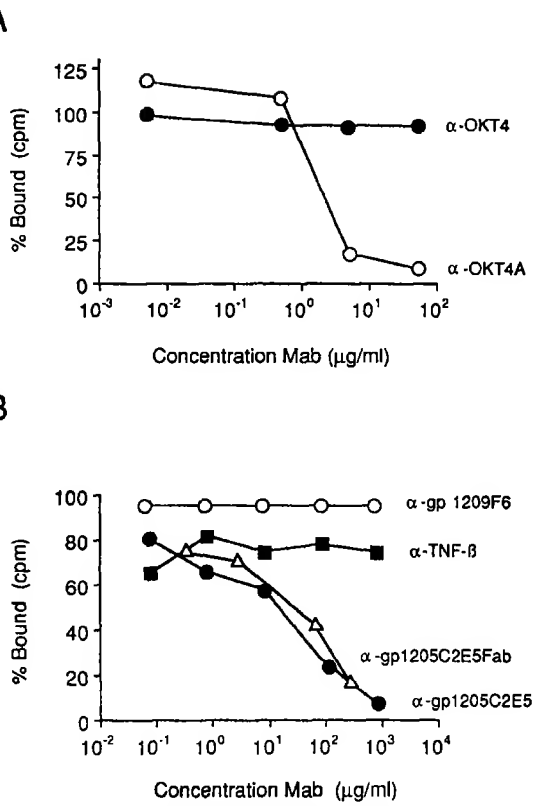
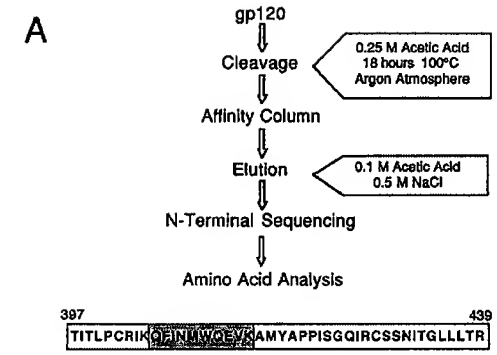


Figure 4. Blocking of the gp120–CD4 Interaction by OKT4 and Anti-gp120 Antibodies

(A) shows the results of adding increasing amounts of either the OKT4 or the OKT4a CD4 monoclonal antibodies to the I^{125} gp120–CD4 binding assay shown in Figure 3. As can be seen here, the OKT4 antibody is ineffective in blocking this interaction whereas OKT4a blocks the interaction, in agreement with previous studies on virus infectivity and native gp120 blocking (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et al., 1985, 1986a, 1986b). (B) shows the results of incubating increasing quantities of three monoclonal antibodies in the I^{125}-gp120-CD4 binding assay. As can be seen in this panel, the control, non-gp120 monoclonal antibody (anti-TNF-β), and an anti-gp120 monoclonal antibody (9F6) are completely ineffective in blocking the interaction between gp120 and CD4. The figure also illustrates that both the complete 5C2E5 monoclonal antibody and the Fab fragment of this monoclonal antibody block this interaction effectively.



Amino acid	N	T	S	E/Q	P	G	A	C	V	M	I	L	Y	F	K	R
Predicted	2	4	3	4	3	3	2	2	1	2	6	4	1	1	2	3
Observed	3.0	4.0	2.8	4.2	3.6	3.1	1.9	0.7	1.4	1.5	5.3	3.8	1.0	1.6	2.1	2.6
	3.0	3.9	2.8	4.2	3.6	3.1	1.9	0.8	1.4	1.5	5.2	3.8	1.0	1.6	2.1	2.6

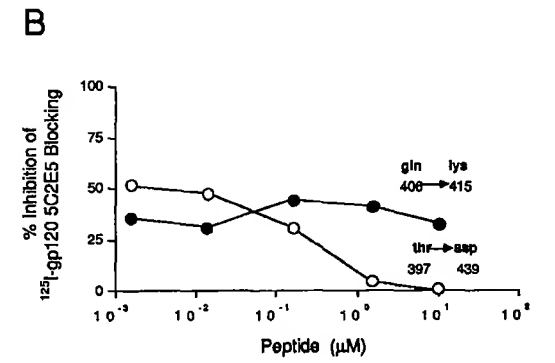


Figure 5. Purification of the gp120 Epitope That Binds to the 5C2E5-Blocking Monoclonal Antibody

(A) Purified gp120 was treated with 0.25 M acetic acid for 18 hr at 110°C under Argon. The material was neutralized and either passed directly over a 5C2E5 immunoaffinity column or first reduced with β-mercaptoethanol and then passed over the column. The bound material was eluted and analyzed by both amino acid analysis and N-terminal sequencing. The figure also shows a tryptic peptide from residues 406 to 415 that binds to the 5C2E5 column and further delineates the 5C2E5-binding epitope. (B) shows that increasing amounts of the isolated aspartate-cleaved 44 amino acid 5C2E5-bound peptide are able to prevent effectively the inhibition of gp120–CD4 binding by 5C2E5, whereas the 10 amino acid tryptic peptide is not.

body may have a critical role in CD4 binding. This interpretation seems especially compelling in view of the absence of blocking ability for the majority of murine gp120 monoclonal antibodies that we tested. To identify the 5C2E5 binding site, an immunoaffinity column was utilized to enable the isolation of the epitope recognized by this antibody. Purified gp120 was treated under mild acid conditions previously shown to specifically cleave proteins after aspartic acid residues (Ingram, 1963). Either disulfide-reduced or nonreduced acid-treated gp120 was passed over the immunoaffinity column, and the peptides that bound to the column were eluted. Amino-terminal sequence analysis of the eluted material revealed that the same unique peptide was bound when either the reduced or nonreduced gp120 hydrolysate was passed over the column (Figure 5). The sequence of this peptide was found to map to a relatively conserved region of gp120 beginning at threonine residue 397 of the mature (i.e., signal sequence minus) gp120 of HIV (Gallo et al., 1984; Ratner et al., 1985; Muesing et al., 1985; Barin et al., 1985) and ending at arginine residue 439 of this protein. The 44 amino acid peptide contains the last two cysteines present in gp120, which appeared to be disulfide-bonded to each other as the same peptide was isolated from both reduced and nonreduced acid gp120 hydrolysates. To define the 5C2E5-binding epitope further, a trypsin digest of gp120 was analyzed by the same procedure. In this case, a peptide from glutamine residue 406 to lysine residue 415 was bound to the column (Figure 5). Mapping of the epitope recognized by both the 5C2E5 monoclonal antibody and the other blocking monoclonal antibody, 7F11, was confirmed by utilizing a library of random gp120 gene fragments inserted into the λ gt11–derived expression vector. This analysis revealed that the same approximate region of gp120 is recognized by both antibodies (D. Dowbenko and L. Lasky, unpublished data). In summary, these results suggest that binding of a monoclonal antibody to a region of mature gp120 between residues 397–439 appears to prevent the interaction between the viral glycoprotein and CD4.

As a further control, we tested the ability of the isolated 44 amino acid peptide to inhibit the blocking of the I^{125} gp120–CD4 interaction by the 5C2E5 monoclonal antibody. Figure 5B shows the effect of increasing amounts of the affinity-purified peptide in the presence of a constant (50% blocking) amount of the 5C2E5 blocking antibody in the gp120–CD4 binding assay. The peptide completely inhibited the ability of the 5C2E5 antibody to block the interaction between gp120 and CD4. By contrast, the 10 amino acid tryptic peptide that bound to the 5C2E5 column was ineffective in blocking the inhibition of the antibody, suggesting that the affinity of this small peptide for 5C2E5 may have been insufficient for inhibition of the gp120–CD4 interaction.

Mutagenesis of the 5C2E5-Binding Epitope

To investigate further the role of the 5C2E5 epitope in the gp120–CD4 interaction, alterations were introduced into gp120 by in vitro mutagenesis, and the resulting mutant polypeptides were tested for their ability to bind the CD4 receptor. Figure 6 summarizes the sequence diversity found among the various HIV-1 isolates sequenced to date in the region containing the 5C2E5 epitope. This region is characterized by three highly conserved segments (amino acids 402–406, 416–423, and 430–439) interspersed with relatively divergent sequences (amino acids 397–401, 407–415, and 424–429) and contains the last two cysteine residues (402 and 429) of gp120 as well as one potential N-linked glycosylation site (asparagine 431).

The first mutation in this region was constructed by deleting 12 amino acids (residues 410–421) positioned equidistant from the two conserved cysteine residues (Figure 6). The deletion removes a small region of nonconserved and a small region of conserved amino acids from this part of the glycoprotein. CHO cells expressing and secreting this mutant polypeptide (Δ410–421) were ana-

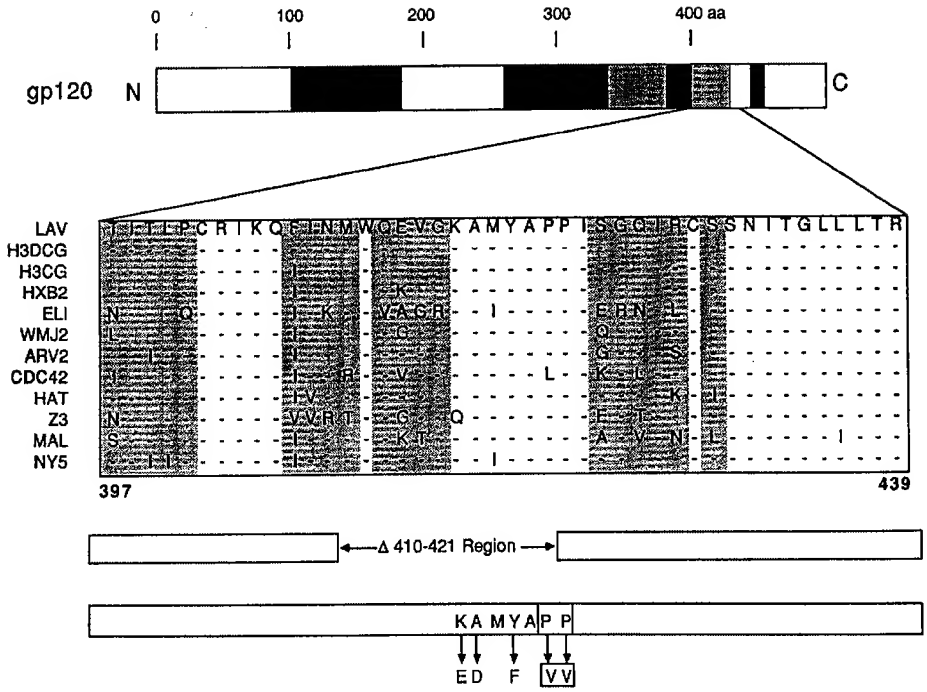


Figure 6. Sequence Diversity in the 5C2E5-Binding Region and Location of the gp120 Deletion and Point Mutants

The top of the figure illustrates the nonconserved (dark), highly conserved (light), and moderately conserved (shaded) regions of gp120 as determined by comparing the protein sequences of all of the published viral sequences (Sanchez-Pescador et al., 1985; Muesing et al., 1985; Starcich et al., 1986; Alizon et al., 1986; Willey et al., 1986; Desai et al., 1986). Also shown is an expanded version of the 5C2E5-binding region showing the microheterogeneity in sequence divergence that is found within this area. The locations of the Δ410–421, lys_{416}–glu, ala_{417}–asp, tyr_{419}–phe, and $pro_{421,422}$–val mutations are also illustrated at the bottom of the figure. The amino acid numbering is for the HTLV-III$_b$ strain of HIV (Gallo et al., 1984; Ratner et al., 1985; Muesing et al., 1985) and begins at the N terminus of the mature gp120 molecule (Barin et al., 1985).

lyzed by immunoprecipitation and Western blot analysis. The mutant protein produced by these cell lines appeared to be indistinguishable from the nonmutant antigen used in the above studies by radioimmunoprecipitation (Figure 7). Western blot analysis revealed that the Δ410–421 protein, unlike the wild-type gp120 molecule, was not recognized by the 5C2E5 monoclonal antibody consistent with the assignment of the 5C2E5 epitope (data not shown). These results suggest that the deletion mutant is similar to the wild-type glycoprotein by the criteria of molecular weight, glycosylation, and anti-gp120 polyclonal antibody reactivity, but that the epitope recognized by the 5C2E5 blocking antibody is not present in the mutant.

Coimmunoprecipitation analysis was carried out to compare the CD4-binding properties of the ^{35}S methionine–labeled Δ410–421 mutant and wild-type gp120 polypeptides. Following incubation with detergent-solubilized CD4, the formation of gp120–CD4 complex was assessed by coimmunoprecipitation with the OKT4 or OKT4a monoclonal antibodies. As Figure 7 illustrates, the wild-type glycoprotein showed a high degree of binding to the CD4 antigen, whereas the Δ410–421 deletion mutant displayed barely detectable binding to the receptor. Competition binding experiments using the iodinated gp120–CD4 binding assay described above gave identical results, with the wild-type glycoprotein effectively competing and the deletion mutant unable to compete for binding of the iodinated gp120 to CD4 (data not shown). These results thus demonstrate that a small deletion in this region has a profound effect on the ability of gp120 to bind to CD4.

To analyze further the role of conserved amino acids deleted from the Δ410–421 mutant, single or double amino-acid substitutions were created by in vitro mutagenesis (Figure 6). These changes were designed to change the charge of an amino acid (lys_{416} to glu, ala_{417} to asp), to remove the capacity for hydrogen bonding (tyr_{419} to phe), or to alter potential secondary structure by removing a predicted β turn sequence ($pro_{421,422}$ to $val_{421,422}$).

Figure 7 summarizes the coimmunoprecipitation behavior of these various substitution mutants. Only one of the mutant polypeptides, ala_{417} to asp, had a detectable effect on the ability of gp120 to bind to CD4 in this assay. This mutant protein appeared to bind weakly to CD4 compared to wild-type or other mutant proteins. These results suggest that the presence of an alanine residue or, alternatively, the absence of a negatively charged residue at this position is important for binding to CD4, and further support the suggestion that this region of gp120 directly interacts with CD4. The results also seem to indicate that other point mutations in this region do not adversely affect the gp120–CD4 interaction, suggesting that some degree of sequence diversity can be tolerated in this region of the glycoprotein.

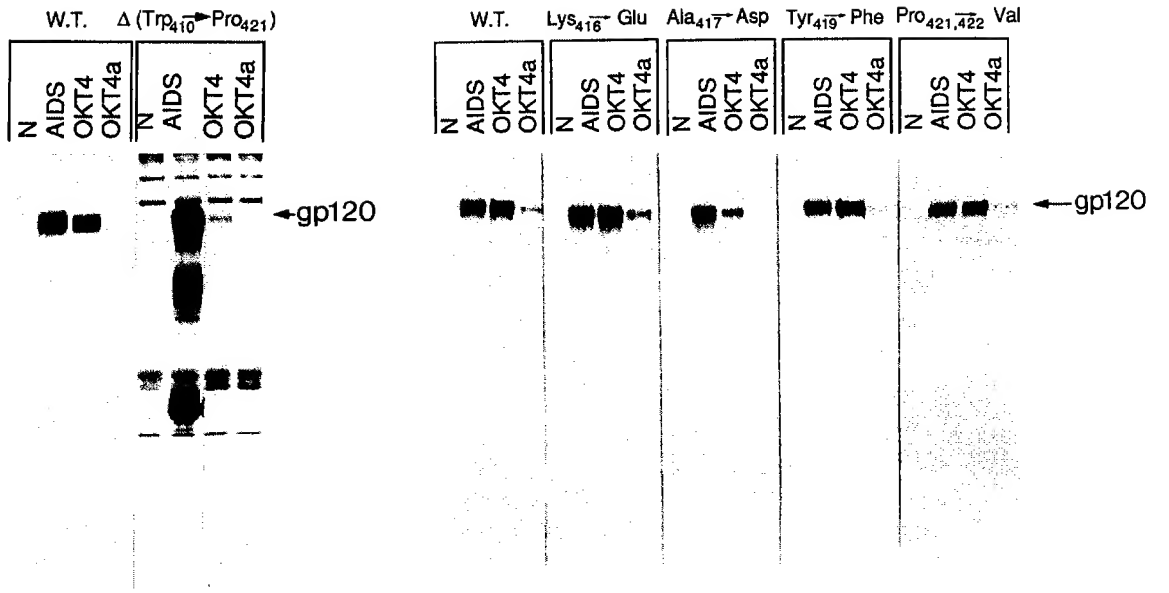


Figure 7. Coprecipitations of Wild-Type gp120, Δ410–421 gp120, and Various gp120 Point Mutants with Anti-gp120 Polyclonal Sera, OKT4 Monoclonal Antibody, and OKT4a Monoclonal Antibody

Expression plasmids containing the wild-type or mutant forms of secreted gp120 were transfected into cultured mammalian cells (Eaton et al., 1986) and the HIV glycoproteins were labeled with S^{35} methionine. The secreted antigens were mixed at 4°C with CD4 detergent solubilized from CHO cells expressing the protein, and the resultant complexes were immunoprecipitated with either the OKT4 or OKT4a monoclonal antibodies. Total immunoprecipitable materials were detected with a high-titer anti-gp120 antiserum from an HIV-infected individual (AIDS). Control immunoprecipitations were done with serum from an uninfected individual (N).

The decreased binding observed for the ala_{417} to asp mutant was further analyzed by competition experiments. A constant amount of ^{35}S-labeled wild-type or ala_{417} to asp mutant gp120 was incubated with CD4 in the presence of increasing amounts of unlabeled wild-type gp120, after which the complexes were radioimmunoprecipitated with polyclonal anti-HIV antisera, OKT4, or OKT4a antibodies. As seen in Figure 8, binding of the ala_{417} to asp mutant gp120 to CD4 was completely blocked upon incubation with 0.3 μg/ml of unlabeled, wild-type gp120, whereas blocking of the wild-type glycoprotein was only attained at a higher level of cold gp120 (1.5 μg/ml). These results confirm that the affinity of the ala_{417} to asp mutant for the CD4 receptor is significantly lower than that found for the wild-type protein, and demonstrates that the substitution of an aspartic acid residue at this position of gp120 has a profound effect on the affinity of gp120 for CD4.

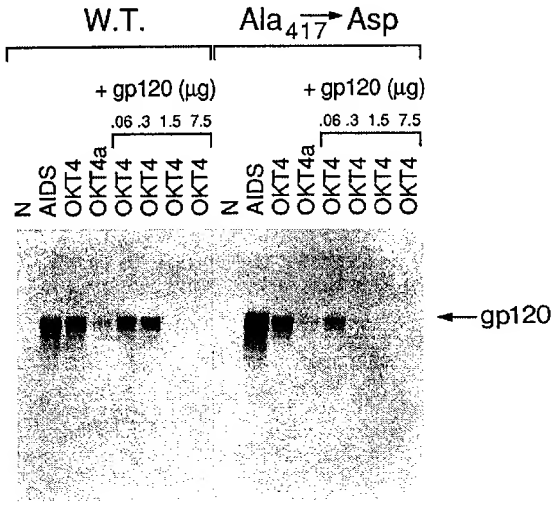


Figure 8. Competition of Wild-Type and Ala_{417}–Asp gp120 for Binding to CD4

S^{35}-labeled wild-type (W.T.) or ala_{417}–asp gp120 were incubated with detergent-solubilized CD4 in the presence of increasing quantities of unlabeled, purified wild-type gp120. The complexes were immunoprecipitated with uninfected (N), HIV-infected (AIDS), OKT4, or OKT4a antibodies and analyzed by SDS–polyacrylamide gel electrophoresis.

Discussion

The data reported here represent the initial characterization of a region of gp120 that appears to be critical for binding to the HIV-1 receptor, CD4. While these results were obtained for a soluble form of the major viral envelope polypeptide, two observations suggest that this interaction is indeed representative of virus–receptor binding. First, the ability of CD4-specific monoclonal antibodies to coimmunoprecipitate the gp120–CD4 complex or to inhibit the binding of these two recombinant proteins mimics results obtained previously for the interaction between native virus and CD4-positive T lymphocytes and T cell lines (Dalgleish et al., 1984; Klatzmann et al., 1984; McDougal et

al., 1985, 1986a, 1986b; Maddon et al., 1986). Second, the high dissociation constant determined for binding of soluble gp120 to CD4 is comparable to those found for other virus–receptor interactions. For example, the dissociation constant for reovirus and its receptor on mouse L cells is 3×10^{-9} M (Armstrong et al., 1984), while the affinity of hepatitis virus binding to its receptor is 1×10^{-9} M (C. Peebles, personal communication). In addition, the binding constant measured here for the gp120–CD4 interaction must be considered a minimum for several reasons. The use of a soluble form of gp120 may give a lower binding constant than that found using gp120 bound to the virus, as the glycoprotein is normally found in association with the transmembrane antigen gp41 on the viral surface (Weiss, 1986). Additionally, the gp120 utilized in these studies is, in fact, a deletion mutant lacking the amino-terminal 30 amino acids found in the mature virally encoded glycoprotein (Lasky et al., 1986; Barin et al., 1985). Included within this missing amino-terminal region is one of the invariant cysteine residues found in all HIV-1 strains sequenced to date (Starcich et al., 1986; Alizon et al., 1986; Willey et al., 1986). The observation that this version of gp120 binds with high affinity to CD4 thus demonstrates that the amino-terminal 30 amino acids of mature gp120 are not indispensable for its interaction with CD4.

The broad range over which the 5C2E5 monoclonal antibody blocks the interaction between gp120 and CD4, the high level of this antibody necessary to block binding, and the inability of this monoclonal antibody to neutralize virus infectivity efficiently (J. Groopman, personal communication) may be related to the observation that although most individuals infected with HIV-1 show some level of virus-neutralizing antibodies, the titers are usually quite low (Robert-Guroff et al., 1985; Weiss et al., 1985, 1986). This may reflect the need for neutralizing antibodies to compete with a higher-affinity interaction between the viral major external glycoprotein and CD4. Similar low titers have been achieved by vaccination of animals with various recombinant gp120 polypeptides (Lasky et al., 1986; Putney et al., 1986). Thus the virus may have evolved mechanisms whereby only low titers of antibodies are directed against CD4 interaction sites, so that the virus may effectively escape immunosurveillance. For example, in the case of picornaviruses, the receptor-binding site(s) may be buried in a cleft within the viral attachment protein that is unavailable for antibody binding or generation (Hogle et al., 1985).

The mechanism of HIV-1 entry into the cell is still unknown. Viruses usually enter cells by an endocytotic pathway or by the interaction of fusogenic proteins on the viral surface with the cellular plasma membrane (Choppin and Scheid, 1980). The results shown in Figure 1 suggest that gp120 is bound to CD4 receptors expressed on the surface of CHO cells and was endocytosed into the cell. Interestingly, attempts to infect CHO/CD4 cells (C. Hansen, personal communication) as well as other nonhuman cell lines expressing CD4 (Maddon et al., 1986) have been unsuccessful. The finding that many nonpermissive CD4-negative cell lines are able to produce virus after transfection with infectious HIV-1 proviral genomes suggests that this block to infection may occur prior to or during entry of the virus into the cell (Levy et al., 1986). Thus the inability to infect CHO cells expressing CD4 seems to indicate that the initial stages of HIV infection are more complex than mere binding of the virus to the receptor. The analysis of a potential glycosylation mutant of gp120 (R. Willey, D. Smith, T. Theodore, L. Lasky, D. Capon, and M. Martin, submitted) provides evidence that infection may indeed involve aspects distinct from binding to the receptor. These may include the ability of the fusogenic domains of the viral envelope protein to function as well as the uncoating of the virus into the cytoplasm of infected cells (Willey et al., submitted).

One potential explanation for the inability of the Δ410–421 gp120 mutant to bind to the CD4 receptor is that this deletion, while small, might have profound effects on the overall structure of the polypeptide. In the absence of three-dimensional structure data for gp120, it is difficult to assess the effects of small deletions or point mutations on the conformation of the protein. However, the high degree of disulfide bond–induced structure (T. Gregory, unpublished data) and high thermal stability of gp120 (McDougal et al., 1986b) seem to indicate that the conformation of the molecule may be relatively insensitive to such mutations. The two cysteine residues flanking the deleted region appear to form a disulfide bond, suggesting that the Δ410–421 deletion may be contained within a loop formed between this cysteine pair, resulting in a decrease in the size of the loop with minimal effects on the rest of the polypeptide. In addition, the fact that the ala_{417} to aspartic acid point substitution within this region showed a marked, although less pronounced, decrease in CD4 binding ability further supports the notion that the effect of these lesions is to impair a critical contact site for receptor binding rather than to alter the overall conformation of gp120.

Although previous expectations have led several investigators to assume that the CD4 interaction sites on gp120 would be highly conserved, the region elucidated here shows only partial conservation. Notably, the highly conserved segments found in this region are interspersed with relatively divergent sequences. This may be indicative of strong selective pressure on the conserved areas to maintain a high degree of sequence preservation. The point mutation studies reported here suggest that not all of the conserved amino acids contained within this region are necessary for high-affinity CD4 binding. It should be noted, however, that the gp120 point mutants produced here may result in a subtle decrease in the affinity constant of the gp120–CD4 interaction that might not have been observed under our experimental conditions. Such changes might have more pronounced effects on the in vivo infectious process. Thus the significant decrease in binding found for the ala_{417} to aspartic acid mutant suggests that this change might impair the ability of the virus to infect CD4-positive cells efficiently.

The sequence heterogeneity found within the gp120 region defined here may bestow important advantages on the ability of HIV to escape immunosurveillance. Thus the observation that this region is able to tolerate such naturally occurring heterogeneity, in addition to that added by the

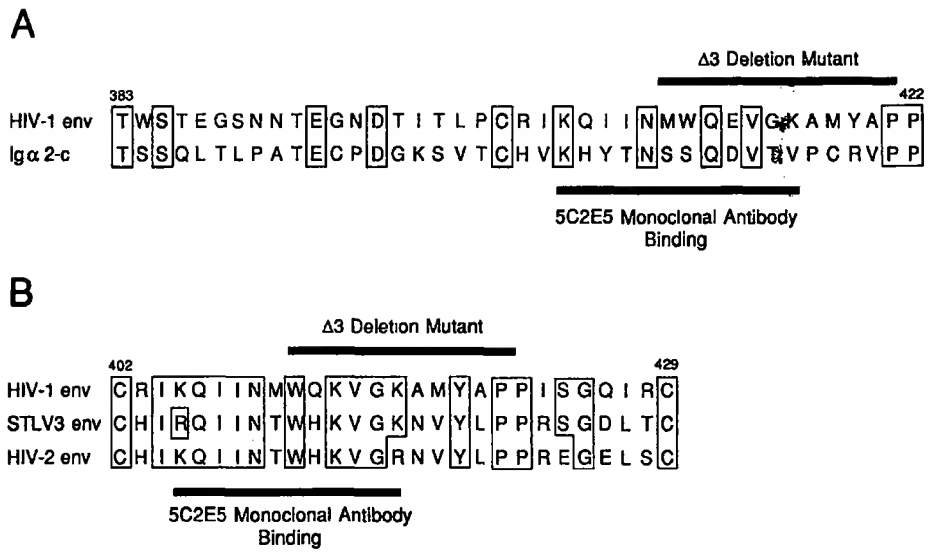


Figure 9. A. Alignment of The 5C2E5 Monoclonal Antibody Binding Domain

(A) Alignment with the Human Immunoglobulin G α2 Heavy Chain (Maddon et al., 1986). (B) Alignment from HIV-1, STLV-3 (Hirsch et al., 1987), and HIV-2 (Guyader et al., 1987).

point mutants described here, yet still bind to the CD4 receptor with apparently high affinity suggests that the virus may have evolved mechanisms to allow for rather significant changes in this site without adversely affecting binding. This would result in viruses that could escape neutralization by mutation of this important region. If this hypothesis is correct, it adds a new complexity to the possible success of an AIDS vaccine. Assuming that this region may potentially be an important neutralizing epitope, this heterogeneity may help to explain the finding that there is not a broad degree of cross-neutralization of a variety of HIV-1 strains when animals are injected with either recombinant (Weiss et al., 1986) or natural gp120 (Matthews et al., 1986).

An interesting aspect of the CD4-binding region elucidated here relates to the proposal by Maddon et al. (1986) that potential CD4-binding domains of gp120 bear limited homology with the immunoglobulin superfamily. Figure 9 shows that one of the two such proposed regions overlaps the segment of gp120 that we have found to be critical for CD4 binding, suggesting that gp120 may indeed interact with CD4 by virtue of its distant relationship to this large superfamily of genes. Maddon et al. (1986) also speculated that a region near the N terminus of gp120 may be part of the CD4-binding site. The secreted gp120 described in this paper is missing approximately half of this region (Lasky et al., 1986), yet still appears to bind with high affinity to CD4. Thus the potential role of this amino-terminal region in CD4 binding remains to be defined. In addition, nucleotide sequences have recently been determined for the envelope glycoprotein genes of STLV-3 (Hirsch et al., 1987) and HIV-2 (Guyader et al., 1987) retroviruses, which also appear to infect CD4-positive cells by binding to the CD4 gene product (Kannagi et al., 1986). It was hypothesized that regions of homology among the gp120 homologs of these lentiviruses may be important for the virus–receptor interaction. Consistent with this suggestion, Figure 9 shows that the region elucidated here is one of the most homologous sequences found in all three otherwise quite divergent external viral envelope glycoproteins.

While the experiments reported here utilized a form of gp120 that was engineered to be secreted from transfected cells, there is evidence that the naturally occurring glycoprotein is weakly associated with the viral surface and is easily shed (Kieny et al., 1986). This observation has interesting implications in light of the high affinity of the soluble gp120 glycoprotein for CD4. Thus the binding to CD4 of gp120 shed from infected cells may constitute one mechanism whereby the virus interferes with normal immune function in the infected individual. The role of CD4 during normal immune responses has been well established (Swain, 1983), and studies using monoclonal antibodies directed against CD4 have demonstrated that T cell helper function is disrupted in the presence of such antibodies (Rogozinski et al., 1984). Thus it is possible that the binding of shed gp120 to CD4 may alter the normal interaction of CD4 with antigen-presenting cells.

It is conceivable that other conserved regions of gp120 contribute directly to CD4 binding (Pert et al., 1986). The strong affinity constant reported here is similar to that of high-affinity antibodies. Recent evidence suggests that several contacts must be made between antibody and polypeptide antigens for a high-affinity interaction to occur (Amit et al., 1986). Furthermore, other highly conserved regions of gp120 might play a structural role in maintaining gp120 in the appropriate tertiary conformation for receptor binding. As shown by Willey et al. (submitted), one such highly conserved region of gp120 may play an important role in viral entry distinct from binding to CD4. Further elu-

cidation of the mechanisms whereby HIV-1 binds to its cellular receptor will, we hope, enable a more rational design of both vaccines and potential therapeutics for HIV-1 infection.

Experimental Procedures

Expression of the HIV-1 gp120 and Human CD4 Proteins

The HTLV-III$_B$ strain of HIV-1 (Gallo et al., 1984) was utilized for isolation of the gp120 gene (Muesing et al., 1985). This protein was expressed in transfected Chinese hamster ovary (CHO) cell lines as a secreted molecule as previously described (Lasky et al., 1986). Briefly, a truncated form of the HIV gp120 gene was expressed under the control of an SV40 early promoter. The expression plasmid also contained a murine dihydrofolate reductase (DHFR) cDNA sequence under the control of a second SV40 early promoter (Simonsen and Levinson, 1983). The DHFR cDNA allowed for the selection and amplification of the plasmid in DHFR-deficient CHO cells. A human CD4 cDNA clone was isolated from a λ gt10 cDNA library derived from the H9/HTLV-III$_B$ cell line (Muesing et al., 1985), utilizing sequence information initially presented by Maddon et al. (1986). The complete CD4 cDNA sequence was expressed under the transcriptional control of an SV40 early promoter in CHO cell lines as a membrane-bound glycoprotein by utilizing a similar SV40-derived vector (Muesing et al., 1987). Cell lines expressing high levels of CD4 were selected and analyzed by immunoprecipitation using OKT4 monoclonal antibodies.

Purification of the Secreted HIV-1 gp120 Glycoprotein

gp120 was purified from supernatants of cell lines expressing high levels of the secreted glycoprotein by immunoaffinity chromatography (Lasky et al., 1986). The isolated gp120 appeared to be >99% homogeneous by SDS–polyacrylamide gel electrophoresis after this purification protocol.

Immunofluorescence of gp120 Bound to CHO Cells Expressing Human CD4

CHO cell lines expressing membrane-bound CD4 were grown on sterile coverslips and were incubated with purified gp120 for 2 hr at 4°C. Human serum from HIV-1-seropositive individuals with anti-gp120 antibody titers was incubated with these cells, after which rhodamine-labeled murine anti-human IgG antibody was added. The temperature was raised to 37°C and the cells were incubated for various periods of time, fixed with acetone-methanol, and observed in the fluorescence microscope.

Coimmunoprecipitation of the gp120–CD4 Complex with OKT4 Monoclonal Antibodies

Purified gp120 was radioiodinated by the lactoperoxidase method as previously described (Garvey et al., 1977) to a specific activity of 0.53 ncl/ng. I^{125}-gp120 was reacted with CHO cells expressing human CD4 in DME media for 1 hr at 4°C. The complexes were solubilized with 1% NP40, after which the solubilized material was immunoprecipitated with the OKT4, OKT4a, or OKT4b monoclonal antibodies (Ortho Pharmaceuticals) and protein A-Sepharose beads. The immunoprecipitates were washed, solubilized in SDS–β-mercaptoethanol buffer, electrophoresed on 10% polyacrylamide gels, and visualized by autoradiography.

Measurement of the gp120–CD4 Affinity Constant

100.0 ng (100,000 cpm) of I^{125}-labeled gp120 was incubated with CHO cells expressing human CD4 for 20 hr at 4°C in the presence of increasing amounts of highly purified unlabeled gp120. The cells were extensively washed and were solubilized, and the radioactive gp120 bound to the cells was counted in a gamma counter. The data were analyzed using the Scatplot program (D. Vandlen, Genentech, Inc.), which fit the data with a least squares analysis and gave the resulting affinity constant. Control cells not expressing CD4 were used to estimate the background binding, which was less than 5% of the specific binding to CD4-expressing CHO cells.

Isolation of gp120 Monoclonal Antibodies That Block the Interaction between gp120 and CD4

Mice were immunized with 30 μg of purified gp120 seven times over a period of seven months. Spleens from the immunized mice were disrupted, fused with NP3x63-Ag8.653 myeloma cells, and selected in HAT medium. Individual wells were analyzed for reactivity with purified gp120 by an ELISA assay. Cells in positive wells were cloned, and the monoclonal antibodies produced by each hybridoma were analyzed in the I^{125} gp120–CD4 blocking assay described above. The assay was initially characterized by adding increasing quantities of either the OKT4 or OKT4a CD4 monoclonal antibodies to the binding assay described above. This gave the expected inhibition of gp120 binding by OKT4a, and lack of inhibition by OKT4. All of the murine gp120 monoclonal antibodies were tested in this assay, and two, termed 5C2E5 and 7F11, were found to block the gp120–CD4 interaction effectively. 5C2E5 Fab fragments were isolated after digestion with papain on an ion exchange column as described by Garvey et al. (1977).

Isolation of the 5C2E5-Binding Epitope by Immunoaffinity Chromatography

The 5C2E5-producing hybridoma was expanded, and the monoclonal antibody produced by these cells was isolated by protein A C14B chromatography. The purified antibody was conjugated to glycerol-coated controlled pore glass (Ohlson et al., 1978). The column was shown to bind and elute the intact purified gp120 effectively. In order to isolate the 5C2E5-binding epitope, purified gp120 was treated with 0.25 M acetic acid overnight at 110°C under Argon. This procedure has been previously shown to cleave proteins at aspartic acid residues by cyclization while leaving the other residues predominantly intact (Ingram, 1963). The cleaved material was either left untreated or was treated with 1 M β-mercaptoethanol. The digest of gp120 was passed over the 5C2E5 immunoaffinity column, washed, and eluted with 0.1 M sodium acetate (pH 3). The eluted material was either subjected to amino acid composition analysis (2.4 μg of peptide) or was sequenced by Edman degradation using an Applied Biosystem gas phase sequenator (12.5 μg of peptide). In addition, the isolated gp120 was treated with trypsin (1% weight per weight) for 2 hr at 37°C, after which the treatment was repeated and terminated by the addition of an excess of soybean trypsin inhibitor. The trypsin-treated glycoprotein was then passed over the 5C2E5 immunoaffinity column. The bound material was analyzed by amino acid composition and N-terminal sequencing as described above. Blocking of the inhibitory activity of the 5C2E5 monoclonal antibody was done by adding increasing amounts of either the acetic acid or trypsin peptides together with a constant amount of 5C2E5 monoclonal antibody to the I^{125} gp120–CD4 binding assay described above.

In Vitro Mutagenesis, Expression of In Vitro Mutants, and Coprecipitation Experiments

A HincII–EcoRV fragment of the gp120 gene containing the region encoding the 5C2E5-binding domain was cloned into the SmaI site of the M13 vector mp18 (Messing et al., 1981). The correct orientation was chosen by dideoxy nucleotide DNA sequencing (Sanger et al., 1977). The following mutagenesis linkers were utilized (5′–3′): Δ410–421, TCCGCTGATGGGGTTTATAAATTG; lys_{416}–glu, GCATACATTGCCTCTCCTACTTCCTG; ala_{417}–asp, AGGGGCATACATATCTTTTCCTACTTC; tyr_{419}–phe, GATGGGAGGGGCGAACATTGCTTTTCC; and $pro_{421,422}$–val, TTGTCCTCGGATCACCACGGCATACATTGC. Mutagenesis was done essentially as described by Zoller and Smith (1982), and the DNA sequence of the mutants was determined. The resultant mutants were excised from the replicative form of M13 by a StuI–HindIII digest, incorporated into a previously described expression vector, and used for transfection onto mammalian cells as previously described (Eaton et al., 1986). S^{35}-labeled mutant glycoproteins were utilized in the gp120–CD4 coprecipitation assay as described above. In this case, a detergent-solubilized form of the CD4 antigen was utilized and the total immunoprecipitable, OKT4-immunoprecipitable, and OKT4a-immunoprecipitable materials were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. Competition of the wild-type and ala_{417}–asp glycoproteins was done by incubating S^{35} methionine–labeled proteins and detergent-solubilized CD4 and increasing amounts of unlabeled, purified gp120 for 1 hr at 4°C, after which the complexes were immunoprecipitated as described above.

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Truncated variants of gp120 bind CD4 with high affinity and suggest a minimum CD4 binding region

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The envelope glycoprotein, gp120, of human immunodeficiency virus type 1 (HIV-1) binds the cellular protein CD4 with high affinity. By deletion we show that 62 N- and 20 C-terminal residues along with the V1, V2 and V3 variable regions of gp 120 are unnecessary for CD4 binding. A 287 residue variant (ENV59), missing those 197 amino acids, binds to CD4 with high affinity. A polyclonal antibody failed to efficiently precipitate ENV59 which is consistent with the loss of immunodominant antigenic structures in the regions deleted. This suggests that ENV59 may have potential as an immunogen, able to elicit antibodies against more conserved regions of gp120. Additionally, complementing co-expressed gp120 fragments as well as a circularly permuted molecule bind CD4, and suggest either that the molecular termini are adjacent in the folded structure, or that an N-terminal region folds into the structure unconstrained by its method of attachment to the rest of the molecule.

Key words: CD4 binding/complementation/deletion mutants/gp120/permuted variants

Introduction

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of acquired immune deficiency syndrome (AIDS) (Barré-Sinoussi, *et al.*, 1983; Popovic *et al.*, 1984). The viral surface glycoprotein gp120, produced by cleavage of a precursor gp160 (Leis *et al.*, 1988), initiates infection by binding the cell surface receptor CD4 with high affinity. This interaction allows efficient infection of CD4-positive cells and directs a major cell tropism associated with HIV-1 infection (Dalgleish *et al.*, 1985; Klatzmann *et al.*, 1985; McDougal *et al.*, 1986). Indeed, soluble recombinant forms of CD4 have been shown to neutralize virus *in vitro* and are being assessed as potential antivirals (Smith *et al.*, 1987; Deen *et al.*, 1988; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Traunecker *et al.*, 1988).

Mutant gp120 molecules engineered *in vitro* indicate that CD4 binding is sensitive to mutations in the C-terminal half of gp120 (Kowalski *et al.*, 1987; Lasky *et al.*, 1987; Linsley *et al.*, 1988; Cordonnier *et al.*, 1989a,b; Olshevsky *et al.*, 1990). Additionally, the epitopes of monoclonal antibodies that specifically block CD4 binding map to the C-terminal C4 domain of gp120 (Lasky *et al.*, 1987; Sun *et al.*, 1989). Many other mutations, particularly in the N-terminal half of the molecule, do not appear to affect CD4 binding. The lack of significant CD4 binding of mutants truncated at the N-terminus, however (Dowbenko *et al.*, 1988), suggests that the binding domain either includes N-terminal regions (Olshevsky *et al.*, 1990; Syu *et al.*, 1990), or that the N-terminus is required for the correct folding of the molecule. Further evidence that correctly folded gp120 is required for CD4 binding derives from the demonstration that several monoclonal antibodies that bind only to 'conformational epitopes' in gp120 block CD4 binding (Ho *et al.*, 1991; Posner *et al.*, 1991).

Here, we describe the CD4 binding properties of a number of deletion mutants of gp120 which were engineered *in vitro* and expressed transiently in COS7 cells. We show that 62 N- and 20 C-terminal residues along with V1, V2 and V3 variable sequence regions of gp120 are unnecessary for CD4 binding. ENV59, a 287 residue variant missing those 197 amino acids and which therefore combines all of the allowed deletions above, binds to CD4 with high affinity. The deleted regions include dominant antigenic structures. Consistent with this, ENV59 failed to precipitate with an anti-gp120 polyclonal antiserum. This result suggests that ENV59, which retains CD4 binding, may have potential as an immunogen. Additionally, complementing co-expressed gp120 fragments and a circularly permuted molecule can bind CD4. These results suggest that an N-terminal region, required for CD4 binding, folds into the structure unconstrained by its method of attachment to the rest of the molecule. The cyclic permutability may additionally, or alternatively, suggest that the molecular termini are adjacent in the folded structure.

Results

N- and C-terminal residues are not required for CD4 binding

Previous reports indicate that while a deletion of 30 amino acids from the mature N-terminus does not affect CD4 binding (Lasky *et al.*, 1987), deletions of 164 N-terminal and 44 C-terminal amino acids abolish binding (Dowbenko *et al.*, 1988; Linsley *et al.*, 1988, respectively). To determine the limits of N- and C-terminal truncations that are allowed before the expressed protein no longer binds CD4, 17 mutants were constructed using the plasmid pCAS and the polymerase chain reaction (see Materials and methods), and expressed in COS7 cells. Expression and CD4 binding (Table I) was then determined by co-precipitations of metabolically labeled protein from cell supernatants with a polyclonal antibody and with soluble CD4, respectively. Eighty-two amino acids, 62 from the N-terminus (construct N93/C491; Figure 1A) and 20 from the C-terminus (N82/C491; Figure 1B) of mature gp120 could be deleted without altering the observed CD4 binding. The removal of two additional amino acids at the N-terminus (N95/C491; Figure 1A) or one more amino acid at the C-terminus

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(N82/C490; Figure 1B) results in a reduction of CD4 binding. Immune precipitations of the cell supernatants using a polyclonal anti-gp120 antibody (in excess) were quantitatively similar, and therefore suggested that there were similar amounts of the mutant proteins in each of the transfected cell supernatants (not shown). Even allowing for the possibility of variations in the antigenicity of the mutant proteins, the observed reduction in the relative amount of protein in CD4 co-precipitates suggests loss of CD4 binding activity. The specificity of binding of the gp120 variants in the CD4 co-precipitations was verified by competition with excess soluble CD4. Non-specifically precipitated material was observed in all experiments, both in the stacking gel (hatched region in Figure 1) and in the resolving gel (~68 kDa protein). However, these materials were not competed away with soluble CD4 and also appeared in control precipitations from cell supernatants which were transfected with carrier DNA only. They were therefore considered artifactual and not relevant to the specific CD4 binding exhibited by the truncated gp120 variants.

Table I. CD4 binding of N- and C-terminally truncated gp120 mutants

Construct[a]	CD4 binding	Construct	CD4 binding
N60/C502	+	N173/C502	−
N82/C502	+	N173/C495	−
N60/C495	+	N100/C502	−
N82/C495	+	N60/C172	−
N60/C491	+	N60/C488	−
N88/C491	+	N82/C488	−
N91/C491	−	N82/C488	±
N93/C491	+	N82/C490	±
		N95/C491	−

[a]The number refers to the N- (N) or C-terminal (C) amino acid included in the mutant protein and corresponds to the HXBCG2 HIV-1 gp120 sequence (Genbank) and includes the signal (Fisher *et al.*, 1985). The + or − refers to whether the mutant bound CD4 (+) or not (−). NB. Mature gp120 comprises 482 amino acids, i.e. residues 30−511.

Some gp120 fragments can complement each other

Although many terminally truncated variants of gp120 were not able to bind CD4 when expressed alone, we have observed that in some instances the co-expression of N- and C-terminal fragments results in molecules that bind CD4 (Figure 1C). We first observed this when an N-terminal fragment consisting of residues 60−172 and a C-terminal fragment representing residues 173−502 were co-expressed. Neither exhibits significant CD4 binding when expressed alone, but when co-expressed they complement each other and are both co-precipitated with CD4 (not shown). The C-terminal fragment, 173−502, corresponds to a V8 proteolytic fragment previously reported to retain indepen-

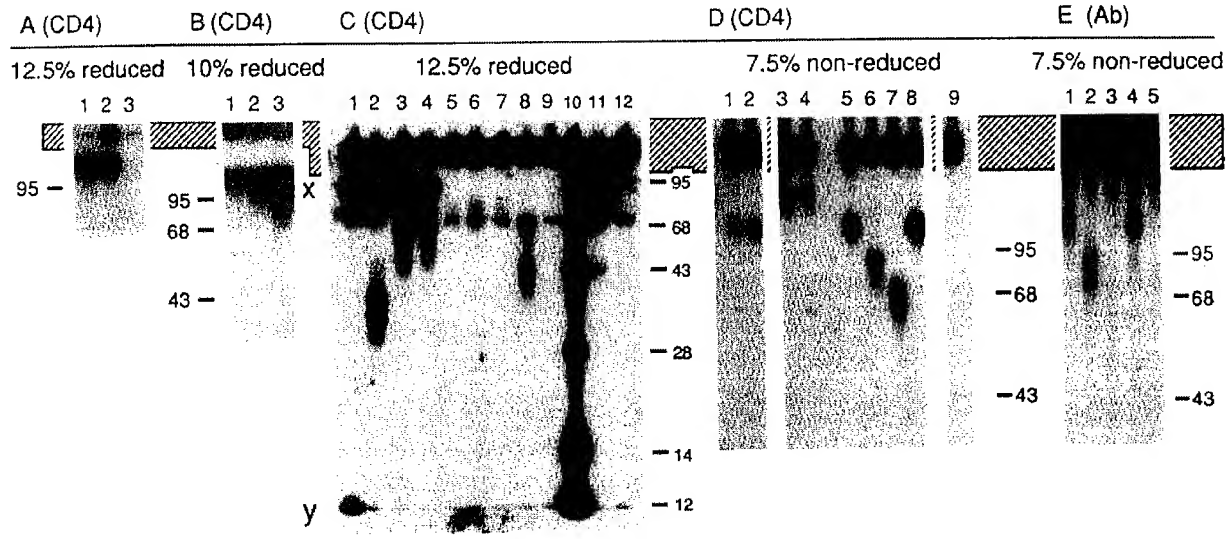

Fig. 1. Co-precipitations of gp120 variants. Shown are several examples of data for the results summarized in Figures 2−5. A−D and E are autoradiographs of SDS polyacrylamide gels of labeled proteins precipitated from cell supernatants by CD4 or polyclonal antibody, respectively. The percent acrylamide and whether proteins were reduced or not is indicated. The hatched region indicates non-specifically precipitated material which remains in the stacking gel. **Gel A**: N-terminal truncations. Lanes 1, 2 and 3 correspond, respectively, to constructions N91/C491 and N93/C491 which bind CD4, and N95/C491 which does not (numbers after N and C refer to N- and C-terminal gp120 amino acids in the expressed protein). **Gel B**: C-terminal truncations. Lanes 1, 2 and 3 correspond to constructions N82/C489 and N82/C490 which show reduced CD4 binding, and N88/C491 which binds CD4 as well as controls. **Gel C**: Co-expression experiments (summarized in Figure 2), e.g. lane 1 shows expt 'a' from Figure 2 demonstrating that fragments 119−502 and 60−118 (x and y in C, respectively) both precipitate with CD4 when co-expressed. Other experiments indicated are as follows (lane in Figure 1: experiments from Figure 2)—1: 1, 2: b. 3: c, 4: d, 5: f, 6: g, 7: h, 8: k, 9: n. Lane 10: molecular weight markers; lane 11: N30/C502 (a CD4 binding control) lane 12: no-DNA control. **Gel D**: Lanes 1−4, covalent association of complementing fragments. experiments (from Figure 2) p, a, c and d correspond to lanes 1, 2, 3 and 4, repectively. Non-reducing conditions show covalent association of the fragments in experiments p and a (lanes 1 and 2) where a protein with a molecular weight approximating to gp120 results (lane 8; N30/C502). For experiments c and d (lanes 3 and 4, a protein of higher molecular weight than gp120 results (lane 8) because of the sequence overlap in the fragments (Figure 2c and d). Lanes 5−9: CD4 precipitations of lane 5: HTT.1 (a circularly permuted molecule, Figure 4); lane 6: HTT.1.V1/V2 (a deletion mutant of HTT.1, Figure 4); lane 7: ENV59 (Figures 4 and 5); lane 8: N30/C502 (CD4 binding control); lane 9: no-DNA control. ENV59 (lane 7) binds CD4 as well as the gp120 (lane 8). **Gel E**: Immune precipitations of lane 1: HTT.1; lane 2: HTT.1.V1/V2; lane 3: ENV59; lane 4: N30/C502; lane 5: no-DNA control. This gel was overexposed to illustrate the poor immune precipitation of ENV59 by the polyclonal antibody.

dent CD4 binding (Nygren *et al.*, 1988). Explanations for the apparent discrepancy with the data of Nygren *et al.* which include evidence that suggests the V8 proteolytic fragment does not in fact retain independent CD4 binding, have been presented elsewhere (Pollard *et al.*, 1991). However, the complementation that occurs between this fragment (residues 173–502) and an N-terminal fragment (residues 60–172) does suggest that N-terminal residues are at least required for the correct folding of gp120, if not directly for CD4 binding itself. Figure 2 summarizes a series of other co-expression experiments using other truncated variants which do not bind to CD4 when expressed alone. Some examples of these experiments are shown in Figure 1C [complementation with purified protein fragments has been observed previously with ribonuclease S and staphylococcal nuclease (Anfinsen, 1973)]. In all cases where complementation was observed, comparison of reducing and non-reducing SDS gels indicated that the complementing fragments always became covalently associated via disulfide bonds (see Figure 1D, lanes 1–4). The disulfide structure of gp120 (Leonard

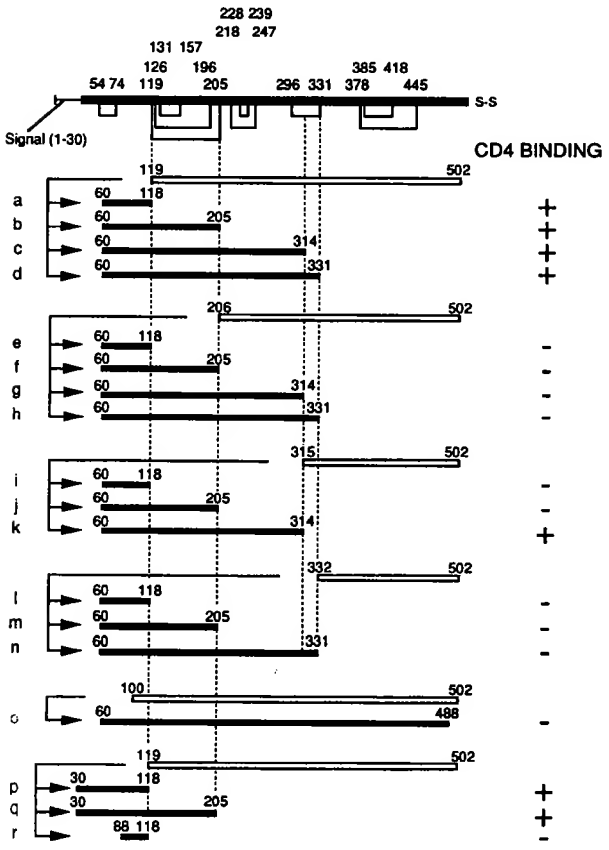

Fig. 2. Co-expression of N- and C-terminal fragments of gp120 sometimes results in complementation. At the top is a diagrammatic representation of the disulfide structure of gp120 (Leonard *et al.*, 1990). Disulfide pairs are indicated below the line. Numbers at the top refer to the 18 cysteines in gp120. Below, indicated diagrammatically, are a series of co-expression experiments performed with a set of N- and C-terminal constructions which produce proteins unable to bind CD4 when expressed alone. Each experiment involved the co-expression of two fragments which are represented by an open and a closed bar. The numbers refer to the terminal gp120 amino acid which is included in the expressed protein. Dashed lines serve to illustrate the relative positions of cysteine residues. The left-hand column indicates CD4 binding: +, co-precipitates with CD4; no strong precipitable band observed.

et al., 1990) indicates that for this to occur in most cases in Figure 2, at least one incorrect disulfide must be made between the complementing fragments relative to the normal gp120 molecule and not affect CD4 binding. In the cases tested, complementation was not observed if the sum of the two fragments contained an internal 'deletion' of gp120 amino acids. Complementation also appears to be sensitive to the relative position of the N- and C-terminus of the two fragments. The failure of fragments 60–331 and 332–502 to complement contrasts with the complementation observed for fragments 50–314 and 315–501 (Figure 2n and k). Similarly fragment 206–502 fails to complement with any of the N-terminal fragments able to complement fragment 119–502, even when no internal 'deletions' would result (cf. Figure 2e–h to 2a–d). The reasons for these different results are unclear, but may be related to the particular cysteines present in each fragment. For example, if covalent association via disulfides is required for complementation to occur, it is possible that for some fragments (i.e. those which fail to complement) intramolecular disulfide bonds are more favorable than intermolecular bonds. Comparison of CD4 precipitations with precipitations with polyclonal antibody gives some indication of the proportion of active CD4 binding molecules (CD4 precipitation) in the total pool (antibody precipitation). When compared to an intact gp120 control, such comparisons suggest that complementation is a relatively efficeint process. In the cases looked at, the active CD4 binding molecules constituted the major proportion (~50%) of the molecules expressed. When complementation was observed, non-reducing gels of the material precipitated by the polyclonal antibody indicated that not all fragments become covalently associated via disulfide bonds. These non-disulfide-linked fragments constituted <50% of the molecules expressed and did not bind CD4.

All of the N-terminal fragments used in the initial experiments began at residue 60 and therefore contained an unpaired cysteine at position 74. Unlike fragment 60–118 (Figure 2a), fragment 88–118 (Figure 2r) did not complement fragment 119–502 despite containing all the gp120 residues required for CD4 binding (see above). These results suggest that, in these cases, the complementing region occurs between residues 60 and 88. As no complementation has been observed without covalent disulfide association, the presence of cysteine 74 between residues 60 and 88 suggests, but does not prove it to be the complementing factor in the region. In these experiments, we were unable to detect any protein of appropriate size by immune precipitation that would correspond to that expected to be produced by the construct N88/C118. However, neither of two independent clones of N88/C118 was able to complement the C-terminal fragment N119/C502, and nucleotide sequence analysis confirmed that the constructions were correct. It therefore seems reasonable to assume that this fragment is expressed and suggests that there are no antibodies in the polyclonal antibody to this small region of the gp120 molecule which can recipitate the expressed protein.

Complementation with formation of inter-fragment disulfides was also observed between fragment 119–502 and the N-terminal fragments 30–118 and 30–205, neither of which contain an odd cysteine (i.e. they contain the 54 and 74 disulfide pair; Figure 2p and q). This indicates that even when all normal pairs of cysteines are present in each fragment, a novel disulfide can arise between fragments, producing a molecule with CD4 binding activity.

CD4 binding can tolerate some internal deletions of gp120 sequences

In the covalently linked complementing fragments described above which contain novel disulfide bonds, the local structure around the incorrect disulfide must be quite different from that in the normal gp120 molecule. Furthermore, when two fragments with large sequence overlap become associated covalently, such as 60–331 and 119–502 (Figure 2d), large structurally aberrant regions must exist and yet do not destroy CD4 binding. It therefore seemed reasonable to investigate internal deletions of the linear sequence of gp120 that correspond to the likely location of these aberrant regions.

A series of deletion mutants were constructed using the polymerase chain reaction (PCR) technique referred to as 'splicing by overlap extension' (Horton *et al.*, 1989). In general, the mutants were engineered so as not to leave unpaired cysteines (Leonard *et al.*, 1990) and to delete sequences corresponding to regions of hypervariable sequence (Modrow *et al.*, 1987), i.e. the V1, V2, V3, V4 and V5 regions or, in one case, a region within domain 3 of the disulfide structure (Leonard *et al.*, 1990) to which antibodies bind but do not block CD4 binding (Ho *et al.*, 1988) (see Figure 3). Sometimes the deletions removed paired cysteines in a region as for deletions V4.2, V1/V2.1, V1/V2.2, Dom3 and V1/V2/Dom3. Other deletions removed the sequences bounded by a disulfide, but retained the cysteines, e.g. V4.1, V3.1 and V3.2.

All deletions reported here which remove regions V4, V5 and the disulfide domain 3 destroyed CD4 binding. However, deletions of the entire V1/V2 region (deletions V1/V2.1 and V1/V2.2) and the V3 region (V3.1 and V3.2) did not result in any observable loss in CD4 binding. The immune precipitation for deletion V3.1, which binds CD4, indicated that it was susceptible to degradation. However, deletion V3.2 which is similar but contains an arginine-proline-glycine-arginine sequence between the cysteines, binds CD4 and is stable (not shown). The original construction intended for the four residue sequence to be glycine-proline-glycine-arginine, a possible β-turn motif, and which corresponds to a conserved sequence in the V3 loop (LaRosa *et al.*, 1990). However, nucleotide sequence analysis of V3.2 revealed a missense mutation, probably resulting from the PCR manipulation, which changes the first glycine or arginine. This sequence, however, retains the potential to form a β-turn structure, suggesting this stabilizes the protein and that the substitution is inconsequential, at least for CD4 binding. The mutants (V1/V2.1 and V1/V2.2) which remove the V1/V2 regions, but still bind CD4, occur in the area where sequence overlap was tolerated in the complementation experiments above, and are probably therefore regions where aberrant structures can be accommmodated in the molecule without inhibiting CD4 binding.

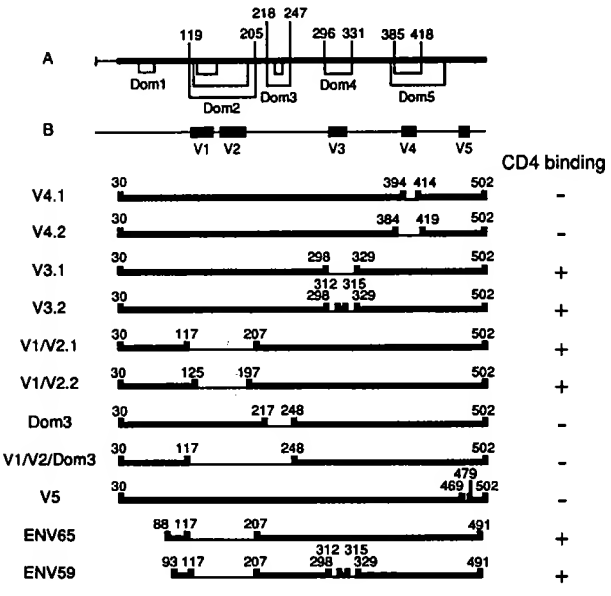

Fig. 3. Deletion mutants of gp120. **Bar A** shows the disulfide structure of gp120 (Leonard *et al.*, 1990). Cysteines relevant to deleted regions are numbered. The disulfide domains are indicated, Dom1–5. **Bar B** shows the position of the five hypervariable regions of gp120 as filled boxes (Modrow *et al.*, 1987). Below are deletion mutants constructed and expressed in COS-7 cells. Numbers refer to the terminal gp120 amino acids which are included in the expressed protein. Thin lines indicate deleted regions and thick lines refer to amino acids included in the mutant protein. The right-hand column indicates CD4 binding: +, specific co-precipitation with CD4; −, no strong precipitable band observed.

The N-terminal, C-terminal and internal deletions that did not affect CD4 binding were then combined to produce a molecule, ENV59 (Figure 3). This variant protein, comprising only 59% of gp120 sequences, is missing the N- and C-terminal truncations of 62 and 20 residues, respectively, the V1, V2 and V3 deletions, four of the nine disulfide bonds and eight of the 24 N-linked glycosylation sites. The mutant ENV59 is efficiently precipitated with CD4 (Figure 1D, lane 7) but was not efficiently precipitated by the polyclonal antibody that was used (Figure 1E, lane 3). Although the polyclonal antibody used was raised against a disparately glycosylated gp120 produced in insect cells, the weak immune precipitation is consistent with the loss in ENV59 of dominant antigenic structures in the N- and C-terminal regions, and in the V1, V2 and V3 variable regions of the molecule.

ENV59 binds to CD4 with high affinity

As a consequence of the inability to precipitate the ENV59 mutant with the polyclonal antibody, we are unable to determine the relative expression of ENV59 to normal gp120. The various gp120 mutants described here, of the size of ENV59 or larger, do not exhibit significantly different expression levels (see Figure 1E). Therefore we reasoned that unless the mutant ENV59 construct expresses at significantly higher levels than the wild-type control, the similar quantities of protein precipitated by CD4 for both (Figure 1D, lanes 7 and 8), suggests that ENV59 binds CD4 with an affinity similar to that of wild-type gp120. However, to ascertain the relative binding affinity of ENV59 more definitively, a series of CD4 precipitations were tried, for both ENV59 and a wild-type gp120 control (construct N30/C502), using a range of CD4 concentrations (200 pM–200 nM). In these experiments, the CD4 co-precipitations were achieved using free soluble CD4 and an anti-CD4 monoclonal antibody which is not cross-reactive with the gp120 binding site and can precipitate gp120/CD4 complexes. This method was employed as a wider range of CD4 concentrations could be tested than was practically possible with the CD4–Sepharose beads. Co-precipitated material was resolved by SDS–PAGE and the resultant autoradiographs analyzed by scanning densitometry. The

densitometric anlayses of these titrated CD4 co-precipitations resulted in the binding curves shown in Figure 4. Both curves clearly plateau, indicating that all the protein able to bind CD4 has co-precipitated. After correction for the expected differences in the specific activities of ENV59 relative to wild-type gp120, the curves essentially superimpose, indicating that the expression levels are similar, as expected above. In addition, half-maximal binding for both occurs in the low nanomolar range, which is in agreement with previous data (4 nM; Lasky *et al.*, 1987) and demonstrates that when all the independently allowed deletions are combined in ENV59, there appears to be little or no loss of CD4 binding affinity.

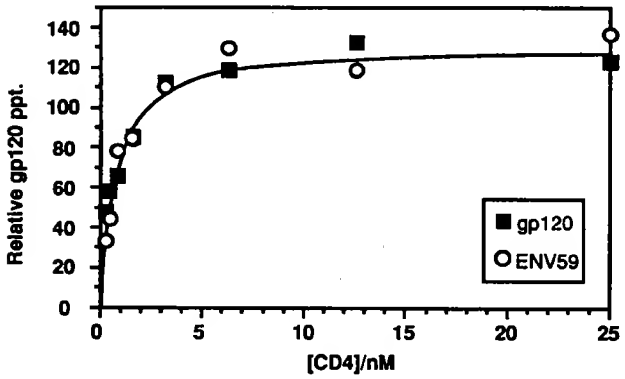

Fig. 4. CD4 binding curves for the mutant ENV59 and wild-type gp120 (residues 30–502). Co-precipitations from metabolically labeled cell supernatants were performed with soluble CD4 at different CD4 concentrations (200 nM–200 pM). The gp120/ENV59–CD4 complexes were precipitated with a CD4 monoclonal antibody (MAbE7) and protein A–Sepharose beads. Precipitated material was resolved on an SDS polyacrylamide gel and the autoradiograph was analyzed by scanning densitometry. Densitometric read-outs were corrected for the expected difference in the specific activity between gp120 (■) and ENV59 (○) (ENV59 values multiplied by 1.8). At 200 nM CD4, the densitometric readings of precipitated bands indicated that for both the mutant ENV59 and the control gp120 a plateau of maximal precipitation had been achieved (the experiment is carried out in antibody excess so that in the region of this plateau the precipitating agents CD4 and antibodies/protein A are in excess). Therefore, half-maximal binding for ENV59 and wild-type gp120 is in the low nanomolar range, which is consistent with previous results (Lasky *et al.*, 1987).

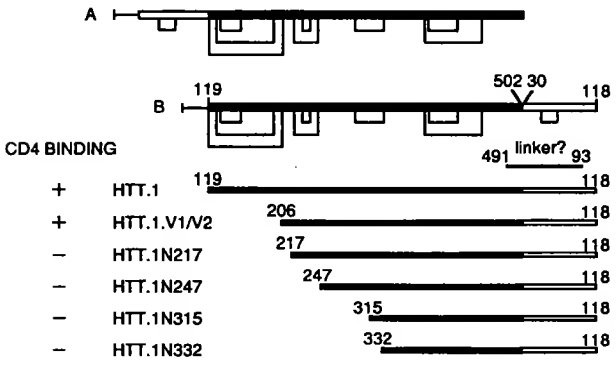

Fig. 5. Circularly permuted gp120 molecules. **A** shows the normal disulfide structure of gp120 as in Figure 2. **B** shows the presumed disulfide structure of a circularly permuted molecule where N-terminal residues 30–118 are moved to the C-terminus adjacent to residue 502. The left-hand column indicates CD4 binding: +, co-precipitates with CD4; –, no strong precipitable band observed. The names of the constructions are referred to on the left. Numbers refer to the terminal gp120 amino acids which are included in the expressed protein.

Circularly permuted variant binds CD4

The 25 amino acid sequence between residues 93 and 117, at least some of which the deletion experiments indicated to be required for CD4 binding (see Figure 6), appears able to tolerate being linked to the rest of gp120 in several ways and still produce a variant with CD4 binding activity. The complementation experiments indicate that it may be linked by an incorrect disulfide involving cysteine 74 (Figure 2A, e.g. fragment 60–118 and 119–502), while the deletion

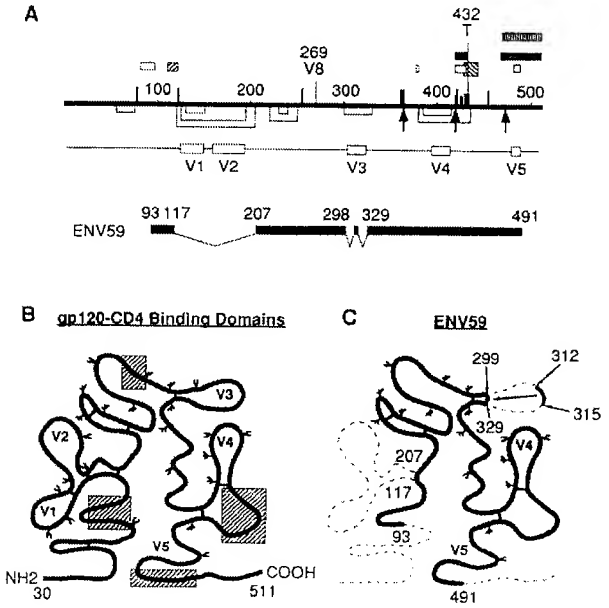

Fig. 6. CD4 binding regions of gp120. (**A**) shows the disulfide structure of gp120 (Leonard *et al.*, 1990). Numbers above line A are amino acid number, unlabeled vertical lines indicate point mutations shown to affect CD4 binding. ▮ (Olshevksy *et al.*, 1990); ▮ (Cordonnier *et al.*, 1989a); ■ (Lasky *et al.*, 1987). Boxes above line A refer to deletions which destroy CD4 binding. □ (Cordonnier *et al.*, 1989b); ▨ (Syu *et al.*, 1990); ▩ (Lasky *et al.*, 1987); ▥ (Cordonnier *et al.*, 1989a); ▩ (Linsley *et al.*, 1988); ▨ (Kowalski *et al.*, 1987). Arrows below the line indicate insertion mutations which affect CD4 binding (Kowalski *et al.*, 1987). V8-269 and T-432 refer to the V8 protease and trypsin sites which are in regions important for CD4 binding (Pollard *et al.*, 1991). The line below shows the hypervariable domains (open boxes). ENV59 is represented as a linear thick line with the deletions represented by thin lines. The sequences retained appear consistent with mutant gp120 molecules previously reported. (**B**) The disulfide structure of gp120 is shown as diagramed by Leonard *et al.* (1990). The amino acid backbone is represented as a thick black line with the N- and C-termini indicated, along with the residue number (i.e. 30 and 511, respectively). The disulfides are illustrated with crossbars (nine in total) and the 24 N-linked glycosylation sites are represented with the symbols Y or Y. The locations of the five hypervariable sequene domains are indicated (V1–V5). The hatched boxes highlight regions which site-directed mutants indicate to be important for CD4 binding (Kowalski *et al.*, 1987; Lasky *et al.*, 1987; Linsley *et al.*, 1988; Cordonnier *et al.*, 1989a, b; Olshevksy *et al.*, 1990; Syu *et al.*, 1990). (**C**) The deletion mutant ENV59. The residues retained in the mutant ENV59 are shown as a thick black line in the context of the disulfide structure shown in A. Deleted sequences are indicated with a thin dotted line contiguous with the thick line. The variable sequence domains which are retained (V4 and V5), as well as the 16 N-linked glycosylation sites also retained in ENV59, are indicated. The numbers refer to the deleted sequences and are the residues retained in ENV59.

mutant V1/V2.1 demonstrates that it can be placed directly adjacent to residue 207 and still bind CD4 (Figure 3). To investigate further the independence of these 25 amino acids with respect to the rest of the structure, we genetically fused them to the C-terminus of gp120 rather than their usual place at the N-terminus. This circularly permuted, head-to-tail gp120 (HTT.1) is able to bind CD4 (Figure 1D, lane 5). The permuted molecule (HTT.1) corresponds to a gp120 where the N-terminal residues 30–118 are placed at the C-terminus adjacent to residue 502 (Figure 5). It is possible that two such molecules could complement each other to create CD4 binding in a way analogous to that observed in the co-expression of fragments 30–118 and 119–502 (see Figure 2p). However, this is unlikely as the HTT.1 molecule does not form the covalent dimers expected if it were analogous to the complementation experiments above (Figure 1D, lane 5). Additionally, an N-terminal deletion of this molecule (HTT.1.V1/V2), akin to the deletion V1/V2.1 of Figure 3, results in another circularly permuted molecule that binds CD4 (Figure 4 and Figure 1D, lane 6). This deletion of the N-terminus of HTT.1 up to residue 206 further suggests this not to be a complementation phenomenon because fragment 206–502 could not be complemented by any N-terminal fragment (see Figure 2). Additionally, this molecule is missing the full set of gp120 amino acids which appear to be a prerequisite for the complementation observed above. Deletion of N-terminal residues beyond 206 in HTT.1 abolished CD4 binding (Figure 4), a result that appears consistent with the inability to delete those residues from gp120, as described above (see Figure 3). The terminal deletion results presented above (Figure 1, footnote 18) would suggest that residues 491–502 and 30–93 in HTT.1 are not required directly for CD4 binding, but may be functioning as a spacer or linker (see Figure 4).

Discussion

The results of the deletion, complementation and other expression experiments presented here indicate that 41% of gp120 residues, i.e. 197 amino acids which include four disulfide bonds and one-third of the N-linked gycosylation sites, are not required for CD4 binding. Consistent with these results, some of the regions identified here as not required for binding have been implicated in other functions of gp120. For example, monoclonal antibodies to the V3 variable region have been shown to inhibit membrane fusion and not to affect CD4 binding (Linsley *et al.*, 1988; Skinner *et al.*, 1988). Additionally, site-directed mutants have indicated that both N- and C-terminal residues of gp120 are important for gp41 association, but not for CD4 binding (Helseth *et al.*, 1991; Ivey-Hoyle *et al.*, 1991).

In every one of the 54 variant gp120 molecules presented, a soluble protein could be precipitated (with either antibody or CD4) from the supernatant of the expressing cell, suggesting that the protein had been secreted and had presumably lost its signal sequence. In the positive cases of CD4 binding, specificity of binding to CD4 was shown by competition with excess soluble CD4. Binding data from mutant gp120 molecules, previously reported, which have suggested regions of gp120 important for CD4 binding appear consistent with the sequences which remain in the CD4 binding variants presented here (summarized in Figure 6a,b). Regions previously identified to be required for binding are also included in the 287 residue variant molecule, ENV59, which retains high-affinity CD4 binding activity (Figure 6). A series of co-precipitations performed at a range of CD4 concentrations, for both ENV59 and gp120, indicate that for both, half-maximal binding occurs in the low nanomolar range. These values are consistent with published data (Lasky *et al.*, 1987) and therefore indicate that ENV59 binds CD4 with an affinity at least approaching that of wild-type gp120. Furthermore, complementation, deletion and, remarkably, cyclic permutation of the sequence indicate that a short ≤ 25 amino acid (93–117) N-terminal sequence apparently required for CD4 binding, or perhaps for correct gp120 folding, appears to be critical, somewhat independent of its method of linkage to the rest of the molecule. The circular permutability may indicate that the N- and C-termini of gp120 are in close proximity to each other in the folded molecule. [The proximity of N- and C-termini enabled functional circularly permuted variants of bovine pancreatic trypsin inhibitor (BPTI) and ribosyl anthranilate isomerase to be produced (Goldenberg and Creighton, 1983; Luger *et al.*, 1989)]. The failure of the polyclonal antibody used here to precipitate ENV59 is consistent with the absence of the antigenic structures in the N- and C-termini, and in the V1, V2 and V3 regions. The use of ENV59 as an immunogen may therefore elicit the production of antibodies against more conserved regions of gp120 which include the CD4 binding domain. Such antigenicity, if exhibited, might be of therapeutic or prophylactic value in the treatment or prevention of HIV-1 infection.

Materials and methods

Plasmid construction

Genetic truncations were engineered using the PCR and introduced into a plasmid pCAS.ENV. Plasmid pCAS.ENV is derived from plasmid pBG381 (Fisher *et al.*, 1988) and contains a gene encoding gp120 from the HXB2 strain of HIV-1 [HXBCG2, Genbank (Fisher *et al.*, 1985)], flanked by unique restriction sites *Mlu*I and *Bst*EII. The gene is engineered to have the CD4 signal sequence at the N-terminus of expressed protein (Fisher *et al.*, 1988). The method of construction results in a stop codon at the C-terminus of the gp120 specific coding sequences. In addition, all mutant proteins expressed using this vector contain the signal sequence and two additional amino acids (Arg Thr; corresponds to the *Mlu*I site) at the N-terminus of the mature secreted protein, in addition to the gp120 sequence. Internal deletion mutants were constructed using the PCR technique referred to as 'splicing by overlap extension' (Horton *et al.*, 1989). In all constructs reported here, cleavage of the signal peptide was demonstrated by immune precipitation of the secreted gp120 variant proteins from the cell supernatant. The plasmid also includes the 3' RNA processing site from the hepatitis B virus genome which enables *rev*-independent expression of gp120 (Emerman *et al.*, 1989). High template concentrations and limited cycles of amplification were used (10 cycles) for all constructions using PCR. This was done to limit the chances of errors caused by the taq polymerase (Horton *et al.*, 1989). Independent duplicate clones were also tested for verification of some results.

Expression of truncated gp120 mutants

All experiments were performed in COS-7 cells in transient expression assays. Plasmid DNA was introduced into the cells by electroporation. Typically, 20 μg each of supercoiled plasmid was used with 380 μg of salmon sperm carrier DNA. Electroporation was performed using a Biorad gene pulser™ with a capacitance extender at 960 mFD, 280 V. The cells were seeded into 6-well dishes to give a confluent monolayer 48 h post-electroporation. Cell proteins were metabolically labeled for 4 h, 48 h post-electroporation, using 1 ml culture medium (DMEM) without methionine, supplemented with [^{35}S]methionine at 100 mCi/ml (NEN, DuPont).

Immune and CD4 co-precipitations

Expression was determined by immume precipitations of material in transfected cell supernatants with a rabbit polyclonal antibody against gp120

and protein A–Sepharose beads (Sigma). The rabbit polyclonal antibody was raised against a recombinant gp120 (HIV-1 strain HXBCG2), affinity purified from *Spodoptera frugiperda* insect cells infected with a recombinant baculovirus (*Autographica californica*). CD4 binding was determined by precipitation with a soluble form of CD4 (Fisher *et al.*, 1988) cross-linked to Sepharose beads, made using methods described by the manufacturers (Sigma). For each precipitation, 10 μl of a 50% suspension of beads was used. Precipitations were incubated overnight at 4°C on a rocking platform. After three washes with 1 ml phosphate-buffered saline (PBS), bound material was eluted from the beads using 2× gel loading buffer and resolved by SDS–PAGE. The specificity of CD4 precipitations was verified by competition with excess free soluble CD4.

Relative affinity measurements

COS7 cells transfected as above with constructs ENV59 or N30/C502 (a wild-type gp120 control) were seeded into T150 flasks to be confluent 48 h post-electroporation. Expressed proteins were metabolically labeled for 4 h in 10 ml culture medium (DMEM) without cysteine, supplemented with [^{35}S]cysteine at 100 mCi/ml (NEN, DuPont). Aliquots (0.5 ml) of labeled transfected cell culture medium were the used in a series of CD4 precipitations, using a range of CD4 concentrations (200 nM–200 pM), for both ENV59 and a wild-type gp120 control (construct N30/C502). For these experiments, soluble CD4 rather than CD4 Sepharose beads was used. The gp120/ENV59–CD4 complexes were precipitated with a CD4 monoclonal antibody (MAb3-7; kindly supplied by Biogen Inc.) and protein A–Sepharose beads. The binding site of the monoclonal antibody MAb3-7 maps to CD4 domains 3 and 4, and is therefore not cross-reactive with the gp120 binding site and does not inhibit gp120 binding. This method was employed, rather than the CD4–Sepharose beads, as a wider range of CD4 concentrations could be tested than was practically possible with the beads. Co-precipitated material was resolved by SDS–PAGE and the resultant autoradiographs analyzed using a scanning densitometer (Molecular Dynamics, ImageQuant™ version 3.0). The densitometric read-outs for the wild-type gp120 control relative to the mutant ENV59 were corrected for the expected difference in specific activity, i.e. 18 cysteines in gp120 compared to only 10 cysteines in the mutant ENV59.

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